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Biophysical Chemistry 97 (2002) 129–138

Biophysical Chemistry

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Structural electrochemical study of hemoglobin by in situ circular dichroism thin layer spectroelectrochemistry

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Received 8 October 2001; received in revised form 14 February 2002; accepted 26 February 2002

Abstract

Secondary and tertiary or quaternary structural changes in hemoglobin (HB) during an electroreduction process were studied by in situ circular dichroism (CD) spectroelectrochemistry with a long optical path thin-layer cell. By means of singular value decomposition least-squares analysis, CD spectra in the far-UV region give two similar α components with different CD intensity, indicating slight denaturation in the secondary structures due to the electric field effect. CD spectra in the Soret band show a R→T transition of two quaternary structural components induced by electroreduction of the heme, which changes the redox states of the center ion from Fe^{3+} to Fe^{2+} and the coordination number from 6 to 5. The double logarithmic analysis shows that electroreduction of hemoglobin follows a chemical reaction with R→T transition. Some parameters in the electrochemical process were obtained: formal potential, $E^{0\prime} = -0.167$ V; electrochemical kinetic overpotential, $\Delta E^0 = -0.32$ V; standard electrochemical reaction rate constant, $k^0 = 1.79 \times 10^{-5}$ cm s^{-1}; product of electron transfer coefficient and electron number, $\alpha n = 0.14$; and the equilibrium constant of R→T transition, $K_c = 9.0$.

Keywords: Hemoglobin; Structural change; Electroreduction; Circular dichroism spectroelectrochemistry; Long optical path thin-layer cell

1. Introduction

Hemoglobin (Hb) is one of the well-known redox [1] and allosteric proteins [2], and has been studied for many years, especially for oxygen affinity. The high-affinity oxy, or R (relaxed), and low-affinity deoxy, or T (tense), states have some differences in quaternary structure, i.e. the relative arrangement of the four subunits, and in tertiary structure, i.e. the conformation of the individual subunits. The quaternary structural changes associated with the R→T transition involves a relative motion between the two αβ dimers making up the tetramer, which corresponds to a rotation by 15° and a translation by 75 pm. The two structures are stabilized by different arrangements of interdimer hydrogen bonds and salt bridges. The oxygen affinity and R→T transition have been previously

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0301-4622/02/$ - see front matter
PII: S0301-4622(02)00045-5

studied [3–5], but the co-operative binding of ligands to hemoglobin and its mechanism remain incompletely understood. Recently, Fourier-transform infrared difference spectroscopy [6] study has shown that there is another kind of conformational transition besides the R→T (quaternary) transition. By measuring the redox formal potential of different ligands along the axial direction of iron in the heme with spectroelectrochemistry, Crumbliss and co-workers suggest a four-fold equilibrium mechanism for the redox reaction and R→T transition [7]. The heme is connected to one of the α helix chains with F8 histidine. The redox reaction of Fe ions in heme ring may have some direct influence on the R→T transition and indirect influence on the secondary structure of the hemoglobin molecule. Among the conformation measurements, circular dichroism (CD) is a powerful tool to study the structure of biomolecules [8,9] in solution with high sensitivity and without limitation of molecular mass. A combination of CD spectrometry with thin layer electrochemistry, called CD thin layer spectroelectrochemistry, has been used to study the electrochemical behavior of biomolecules [10,11]. In the present paper, CD spectroelectrochemistry with a long optical path thin-layer cell (LOPTLC) was used to study the secondary and quaternary structural changes in hemoglobin during electroreduction. By means of singular value decomposition least-squares (SVDLS) analysis [12], more information about the structural changes and mechanism was obtained.

2. Experimental results

2.1. Cyclic voltammetric behavior of Hb

A cyclic voltammogram (CV) of 0.50 mg ml^{-1} Hb in pH 7.21 phosphate buffer solution containing 0.20 M KNO_3 is shown in Fig. 1 in the potential range from 0.50 to −1.00 V at 50 mV s^{-1} scan rate at a glassy carbon (GC) electrode. Hb showed an irreversible reduction peak at approximately −0.35 V (Fig. 1, solid line). The peak decreased during the second scan and became stable. The peak potential was 0.25 V more positive than that of Hb at a Hg electrode [−0.60 V vs. saturated calomel electrode (SCE)] [10]. The CV curves of Hb at a methylene green-modified GC electrode under the same experimental conditions showed a quasi-reversible process (Fig. 2) with $E_{1/2} = -0.167$ V, similar to that of Hb at a methylene blue-modified Pt electrode with a four-electron transfer process [10]. The CV results suggest that the potential range from 0.60 to −1.00 V is suitable to investigate the influence of redox reaction on the quaternary and secondary structures of hemoglobin.

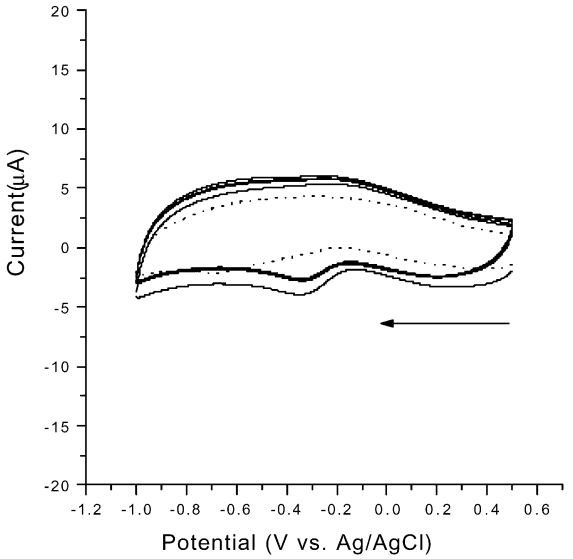

Fig. 1. Cyclic voltammogram of Hb at GC electrode: 0.50 mg ml^{-1} Hb in 10 mM phosphate buffer solution (pH 7.21) containing 0.20 M KNO_3. Scan rate, 50 mV s^{-1} from 0.50 to −1.00 V; the dashed line is the background. Peak potential, −0.35 V.

2.2. CD spectroelectrochemical behavior of Hb in far-UV range

A solution of 0.138 mg ml^{-1} Hb in 10 mM phosphate buffer (pH 7.21), containing 0.2 M KNO_3, was used to perform in situ CD spectroelectrochemistry in the far-UV region. The CD spectra of Hb in the 200–240-nm region changed with applied potential (Fig. 3), showing a typical α helix CD spectrum with two negative Cotton peaks at 208 and 222 nm [8]. The maximum CD spectrum change at 215 nm was approximately

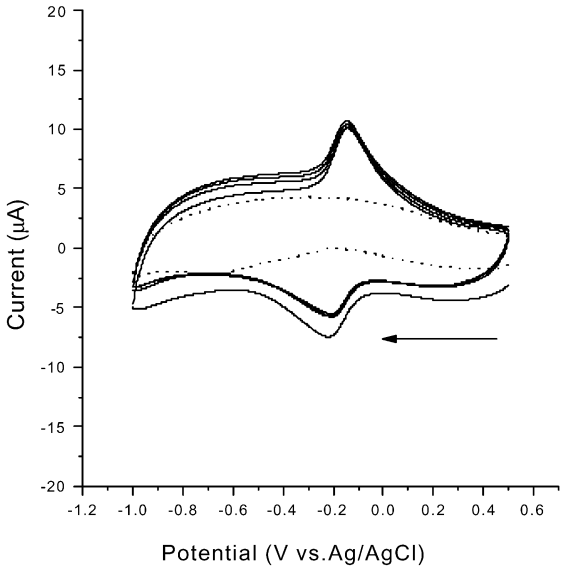

Fig. 2. CV curve of Hb at methylene green-modified GC electrode; the dashed line is the background. Experimental conditions as for Fig. 1. Formal potential, −0.167 V.

13%. By applying SVDLS analysis [12] to the CD spectral data in Fig. 3, two CD spectra of secondary structure components and their fraction distribution with applied potential were obtained, as shown in Figs. 4 and 5, respectively. Both of the two CD spectra extracted (Fig. 4) were identified as α helices with a difference in CD intensity. The pattern of CD spectral changes were very similar to those of thermolytic fragmentation of colicin A occurring during an acid denaturation process [with larger amplitude in neutral solution (pH 7) and smaller amplitude in acidic solution (pH 2) [13]] and to those of a peptide-sandwiched mesoheme [14] interacting with 2,2,2-trifluoroethanol. The one with smaller amplitude was been considered as the mixture of α helix and random coil and identified as partly denatured peptides. The distribution curves (Fig. 5) showed that the two components convert from one to another through a critical point at 0.00 V. When the potential was more positive or negative than 0.00 V, the fraction of the component with larger amplitude decreased, whereas the one with smaller amplitude increased. This implies that the denaturation of hemoglobin

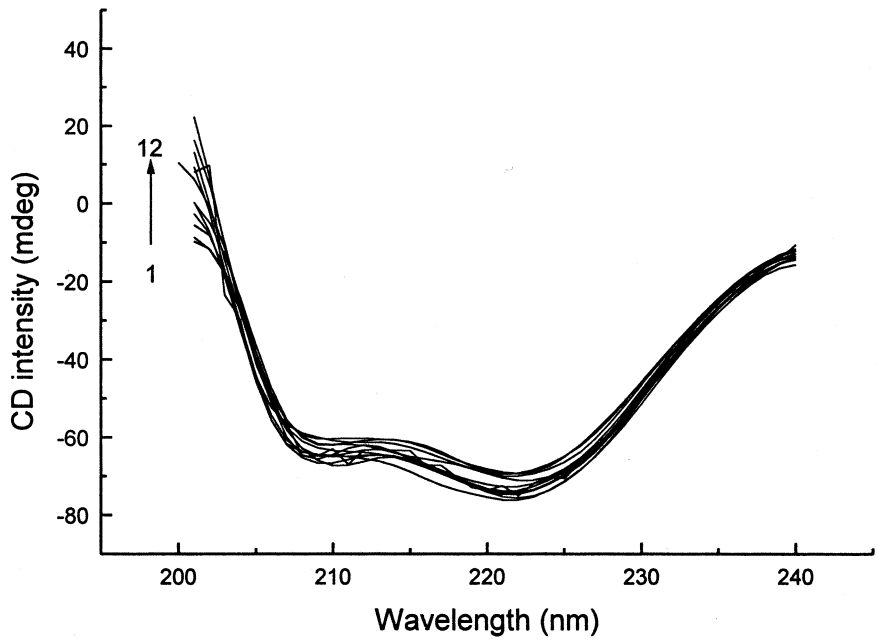

Fig. 3. CD spectra of Hb with varying potential: 0.138 mg ml^{-1} Hb in 10 mM phosphate buffer solution (pH 7.21) containing 0.20 M KNO_3. Time interval, 6 min; applied potential 1→12, 0.5 to −0.6 V, step −0.1 V.

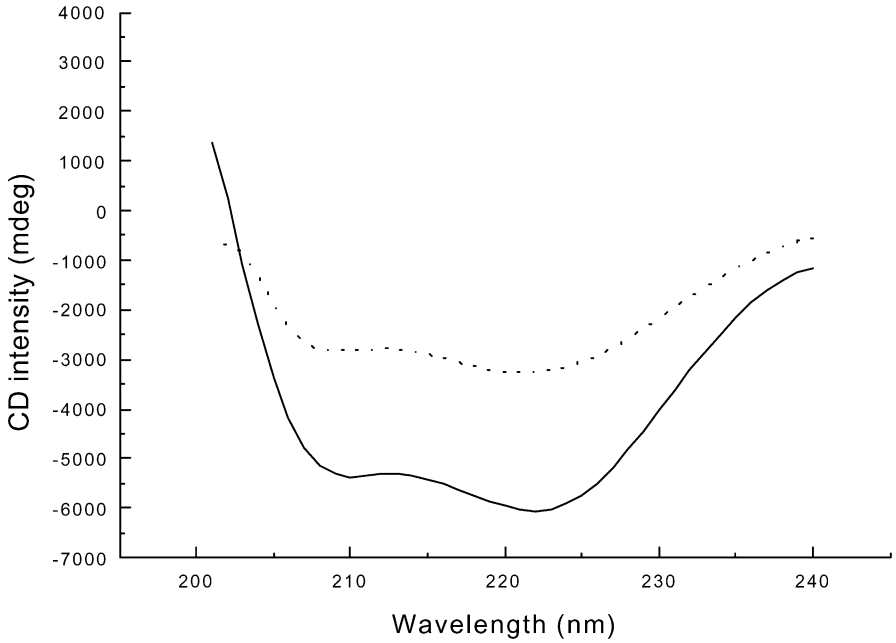

Fig. 4. CD spectra of secondary structures of Hb: solid line, α helix 1; dashed line, α helix 2. The experimental data are from Fig. 3 and were treated by the singular value decomposition least-squares method (SVDLS).

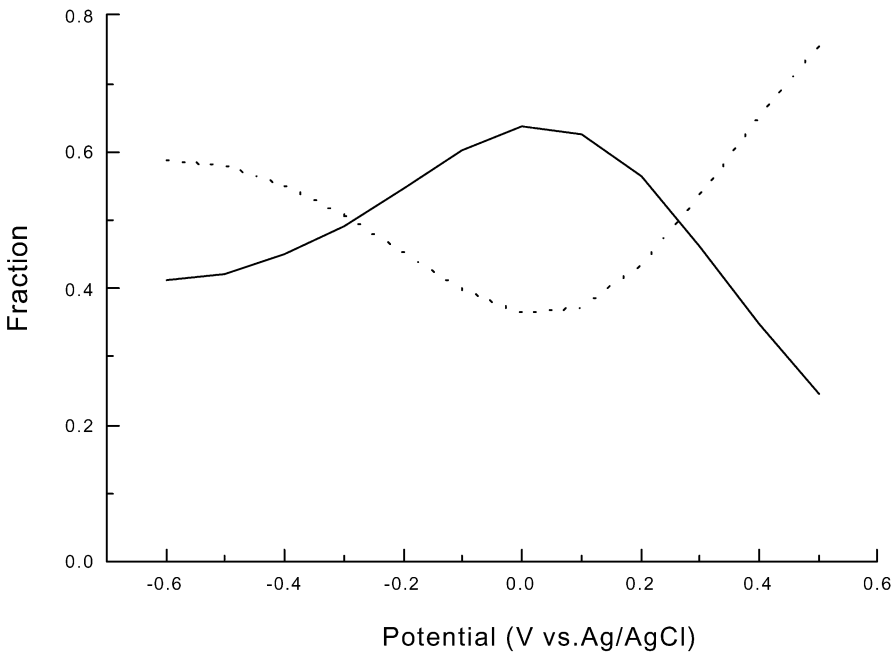

Fig. 5. Fraction distribution of secondary structure of Hb: solid line, α helix 1; dashed line, α helix 2. The experimental data are from Fig. 3 and were treated by SVDLS. The maximum point at 0.00 V means it is an electric field effect.

was influenced by the electric field with both positive and negative sign, and had nothing to do with reduction of the heme ring.

2.3. CD spectroelectrochemical behavior of Hb in the Soret band

A solution of 0.55 mg ml^{-1} Hb in 10 mM phosphate buffer (pH 7.21), containing 0.2 M KNO_3, was used to perform in situ CD spectroelectrochemistry in the Soret band. The CD spectra of Hb in the Soret band showed two positive Cotton peaks at 420 and 380 nm, as shown in Fig. 6. The whole CD spectrum increased for the first few applied potential steps. The peak at 380 nm then continued to increase, whereas the peak at 420 nm decreased. The maximum change in amplitude of the CD spectrum at 400 nm was approximately 18%. The corresponding absorption spectra with one peak at 406 nm were also recorded, as shown in Fig. 7. The absorption peak decreased for the first several potential steps, and then shifted to 417 nm when the applied potential was more negative than -0.50 V. By applying SVDLS to the CD spectra in Fig. 6, two components of CD spectra were obtained, as shown in Fig. 8. One with two peaks at 420 and 380 nm was very similar to that in Fig. 6 at first line, which was related to the oxidized form of Hb (*solid line*); the other (with a very good relationship to the previous one) was related to the reduced form of Hb (*dashed line*). The mirror-image relation of the two CD spectra might be induced by two kinds of environments around the Fe ion, with opposite chirality. The fraction distribution curves of the two CD components are shown in Fig. 9. The fraction distribution curve related to the oxidized form decreased slowly when potential was more positive than -0.30 V and decreased rapidly when potential was more negative than -0.30 V, whereas the one related to the reduced form increased with a similar pattern. The two fraction distribution curves also showed a very good mirror-image relation, with a crossover point at -0.50 V.

3. Discussion

3.1. Secondary structure changes of Hb

Hb is a typical helical protein with four subunits making up the whole molecule with a $\alpha_2\beta_2$ structure; four hemes are connected to one α segment in each subchain through F8 histidine. The cooperation of the four subunits, four hemes and other co-operative binding of ligands determine the quaternary and tertiary structure of the molecule. Besides the α helix conformation, other helices exist, such as π-, γ- and 3_{10}-helices [15]. When only a small part of the α helix conformation changes into a random coil or other kinds of helical conformations, the resulting CD spectrum may retain the original shape but be reduced in intensity. A small intensity change in the CD spectra (maximum is approx. 13%) in Fig. 4 implies that the electrochemical process only slightly influences the secondary structure. The critical point at $E=0.00$ V in the fraction distribution curves (Fig. 5) far away from the $E_{1/2}$ of Hb in the CV curve implies that the secondary structure change has nothing to do with the electroreduction, but coincidences with the charge property of the electrode surface. Hb has an isoelectric point [16] at pH 6.72, and it will be negatively charged in the experimental solution (pH 7.21). When the potential is more positive than 0.00 V, the electrode is positively charged and has a strong electrostatic attraction to the negatively charged Hb molecule. When the potential is more negative than 0.00 V, the electrode is negatively charged and has a strong repulsion to the negatively charged Hb molecule. Thus, the secondary structure changes may be due to the effects of the electric field and partly denatured protein molecules. The positive electric field has stronger influence than the negative one.

3.2. Tertiary and quaternary structural changes and $R \rightarrow T$ transition

Hb, in the absence of allosteric effectors, exists in a $R \leftrightarrow T$ equilibrium and undergoes a transition from the T state to the R state on oxidation. According to the literature [7], the absorption peak at 406 nm in Fig. 7 corresponds to the R state, whereas the absorption peak at 417 nm may be identified as T state, which is 13 nm lower than that in the literature. From X-ray studies [17], the four N atoms in the heme are not really in one plane, but have different angles and bond lengths. The four N atoms of the heme ring, together with

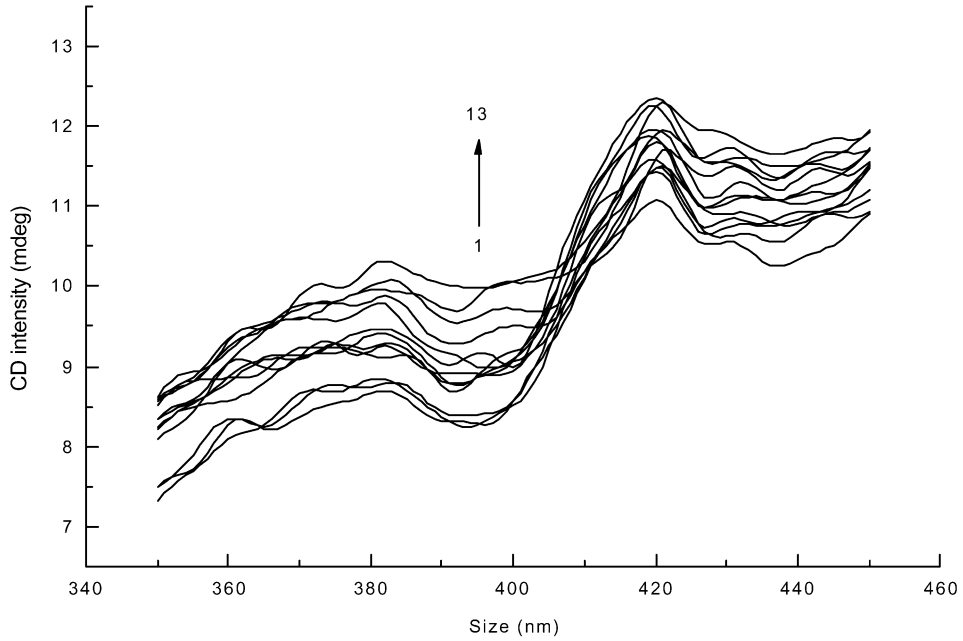

Fig. 6. CD spectra of Hb with different applied potential in the Soret band: 0.55 mg ml^{-1} Hb in 10 mM phosphate buffer solution (pH 7.21) containing 0.20 M KNO_3. Time interval, 6 min; applied potential 1→13, 0.3 to −0.9 V, step −0.1 V.

the ligands along the axis direction, form the chiral environment of the Fe ion. In the T state, there are mainly five co-ordinates (four N atoms from the heme ring and one N atom from F8 histidine) around the Fe ion, which gives a configuration with the Fe atom out of the heme plane approximately 75 pm toward the histidine ligand. In the R state, an oxygen or a water molecule co-ordinates with Fe from the opposite direction of F8 histidine along the molecular axis, so that the Fe atom with six co-ordinates is in the heme plane. During the R→T transition, the Fe atom moves from inside the plane to the outside of the plane, resulting in the chirality around the Fe atom partly changing from left- to right-handed, so that the CD spectra of the R and T states have a very good mirror-image relation, as is the case in Fig. 8. Comparing the fraction distribution in Fig. 9 with CV in Fig. 1, it is evident that the crossover point for the R→T transition (0.50 V) is more negative than the $E_{1/2}$. The electroreduction of Fe^{3+} to Fe^{2+} in heme before the R→T transition implies that electroreduction induces the R→T transition. Many allosteric systems show a R→T transition accompanied by chirality changes of the center ion and mirror-image relations of the CD spectra of T and R configurations. CD spectroscopy is highly suitable for studies in these systems.

3.3. Mechanism and parameters of the electroreduction of Hb

Taking the fraction distribution of the oxidized form of Hb as the concentration, and taking a double logarithm of the inverse of concentration, $\ln[\ln(1/c)]$, according to the previous double logarithmic method [18], Fig. 10 shows one oblique line, along with one line parallel to the potential axis (line with ×××). This type of double plot indicates a typical EC mechanism, i.e. an irreversible electroreduction followed by a chemical reaction. According to the double logarithmic plot and the mechanism suggested in the literature [18], the mechanism of the electroreduction process of Hb can be expressed as:

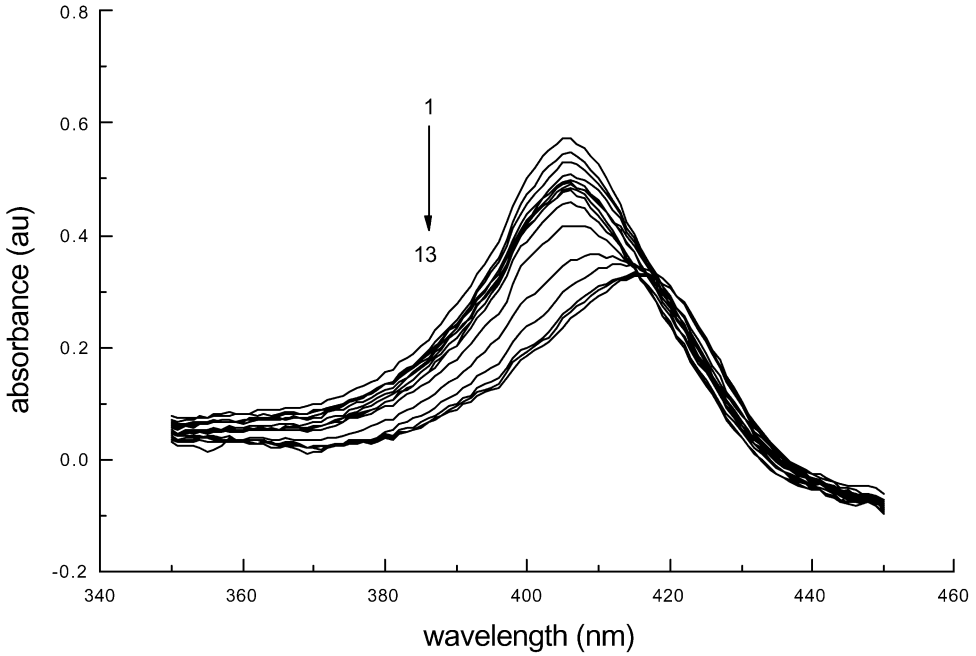

Fig. 7. Absorption spectra of Hb in the Soret band with different potential. The experimental conditions were as for Fig. 6. T state, 408 nm; R state, 417 nm.

$$\mathrm{Hb(III)R} \xrightarrow[+\mathrm{e}]{k^0} \mathrm{Hb(II)R} \quad (1)$$

$$\mathrm{Hb(II)R} \overset{K_c}{\rightleftharpoons} \mathrm{Hb(II)T} \quad (2)$$

This mechanism further proves that the two mirror-image CD spectra correspond to the R and T forms of Hb, respectively, and electroreduction induces the R→T transition. The product of the electron transfer coefficient and the number of electrons, $\alpha n = 0.14$, is obtained from the slope of the oblique line ($y = -2.687 - 5.352E$, $R = 0.88$, S.D. = 0.768, $P = 0.00403$), and the apparent formal potential, $E^{0\prime} = -0.50$ V, is obtained from the peak position in the differential curve (Fig. 10, line with ●●●). Taking $E^{0\prime}$ and αn as known parameters to perform normal non-linear regression, the standard heterogeneous electron transfer rate constant, $k^0 = (1.79 \pm 0.23) \times 10^{-5}$ cm s^{-1}, and the chemical equilibrium constant for Eq. (2), $K_c = 9.0 \pm 0.9$, are estimated with S.D. = 0.114. A similar value of $K_c = 8.1$ can also be estimated from the maximum ratio of the fractions of the oxidized and the reduced form of Hb at −0.80 V (Fig. 9). The K_c value implies that the R state is more stable than the T state in the reduced form of Hb under experimental conditions, which is in accord with the result in [7].

CD spectroelectrochemistry monitors the whole electroreduction process, including Eqs. (1) and (2), so that the apparent formal potential obtained includes three parts: the first part is from the equilibrium formal potential, E^0, which is obtained from CV curve at the methylene green-modified GC electrode as −0.167 V; the second is from the equilibrium constant of the following chemical reaction, which can be obtained from thermodynamic relation of $\Delta G^0 = -nfE$ as $\Delta E^0 = -0.014$ V; and the last is the electrochemical kinetic overpotential, $\Delta E = 0.32$ V.

4. Conclusions

Experimental results show that electroreduction of Hb induces a R→T transition of the quaternary structure, which is found by SVDLS analysis from

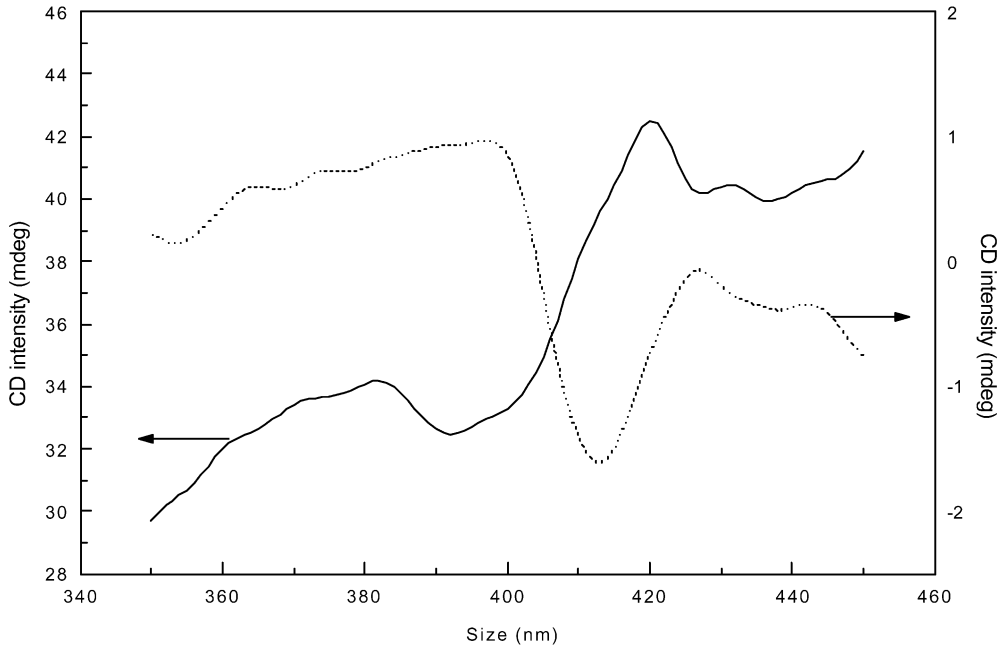

Fig. 8. CD spectra of Hb with different structures: solid line, R state; dashed line, T state. The experimental data are from Fig. 6 and were treated by SVDLS.

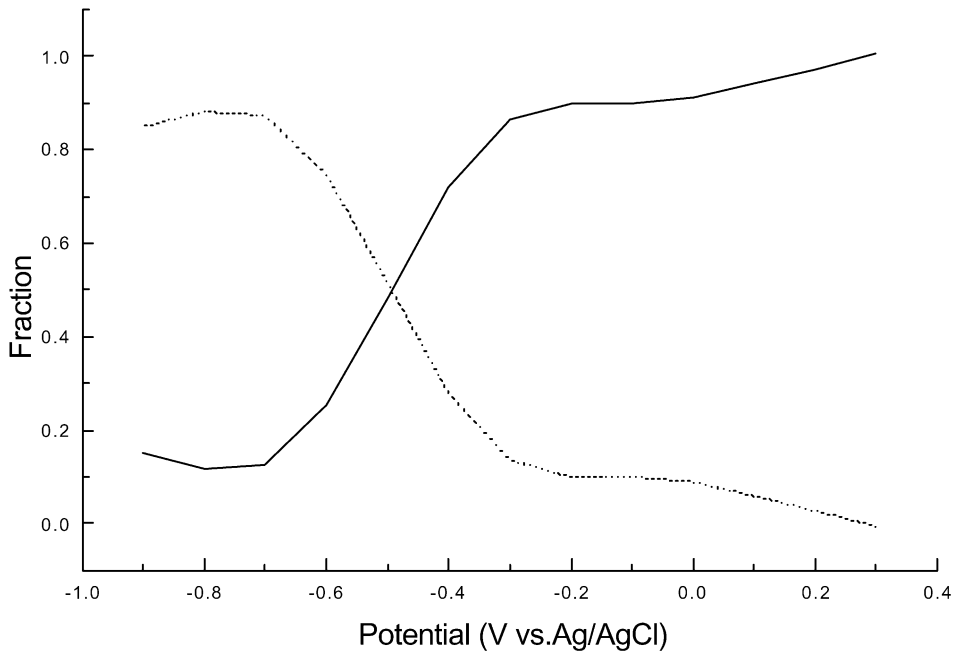

Fig. 9. Fractional distributions of different states: solid line, R state; dashed line, T state. The experimental data are from Fig. 6 and were treated by SVDLS. The two lines cross at −0.50 V, near the apparent formal potential.

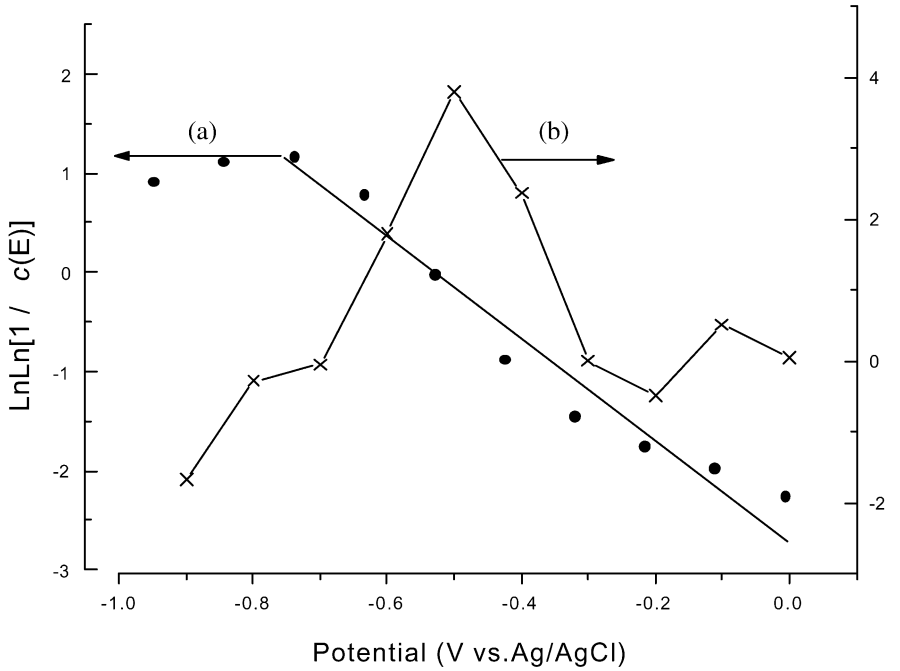

Fig. 10. Double logarithmic plots of oxidized Hb: ××× double logarithmic curve; ●●● differential curve. The experimental data are from Fig. 6. EC mechanism; apparent formal potential, −0.50 V; $\alpha n = 0.14$; $k^0 = 1.79 \times 10^{-5}$ cm s^{-1}; $k_c = 9.0$ by non-linear regression.

dynamic CD spectra for the first time. CD spectroscopy is very sensitive to local chirality changes in biomolecules, and is suitable for studying the T→R transitions of allosteric systems. The secondary structures of Hb are influenced by an electric field to a small extent, with the interaction of charges in the molecule, but not by the electroreduction. The electroreduction process of Hb according to the EC mechanism is followed by the R→T transition. SVDLS and double-logarithmic analysis are very useful tools for extracting information, such as CD spectra of each conformation, fraction distribution curves, the mechanism and some thermodynamic or kinetic parameters of the process, from a complex dynamic data matrix for conformational study of biomolecules.

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