ELSEVIER

Biophysical Chemistry 90 (2001) 1–8

Biophysical Chemistry

www.elsevier.nl/locate/bpc

The electrochemically induced conformational transition of disulfides in bovine serum albumin studied by thin layer circular dichroism spectroelectrochemistry

Yongchun Zhu, Guangjin Cheng, Shaojun Dong*

Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, PR China

Received 13 July 2000; received in revised form 22 November 2000; accepted 27 November 2000

Abstract

The conformational transition of disulfides in bovine serum albumin (BSA) induced by electrochemical redox reaction of disulfides were monitored by in-situ circular dichroism (CD) spectroelectrochemistry, with a long optical path thin layer cell and analyzed by a singular value decomposition least square (SVDLS) method. Electrochemical reduction of disulfides drives the left-handed conformation of disulfides changed into the right-handed. At open circuit, eight of the 17 disulfides were of left-handed conformation. Four of the 17 disulfides took part in the electrochemical reduction with an EC mechanism. Only one-fourth of the reduced disulfides returned to left-handed conformation in the re-oxidation process. Some parameters of the electrochemical reduction process, i.e. the number of electrons transferred and electron transfer coefficient, $n = 8$, $\alpha n = 0.15$, apparent formal potential, $E_1^{0'} = -0.65(\pm 0.01)$ V, standard heterogeneous electron transfer rate constant, $k_1^0 = (2.84 \pm 0.14) \times 10^{-5}$ cm s^{-1} and chemical reaction equilibrium constant, $K_c = (5.13 \pm 0.12) \times 10^{-2}$, were also obtained by double logarithmic analysis based on the near-UV absorption spectra with applied potentials.

Keywords: Bovine serum albumin; Disulfides; Conformational transition; Electrochemical reaction of protein; In-situ thin layer circular dichroism spectroelectrochemistry

* Corresponding author. Tel.: +86-431-568-2801; fax: +85-431-568-5653.
E-mail address: dongsj@ns.ciac.jl.cn (S. Dong).

0301-4622/01/$ - see front matter
PII: S0301-4622(00)00238-6

1. Introduction

Bovine serum albumin (BSA) is a small protein with a single polypeptide chain of molecular weight 69 000, which is cross-linked by 17 disulfide bonds [1]. The molecule also contains one free sulfhydryl group located on the molecular surface; 68% of it is in the free form and 32% of it is complexed with cysteine or glutathione [2]. The two-dimensional structure of the molecule has been determined by Brown [3]. The polypeptide chain is brought into close proximity by two disulfide linkages in eight places; adjacent cysteine residues cannot cross-link because the sulhydryl moieties are located trans to one another. Other interactions, such as hydrogen bonding and van der Waals forces, maintain the secondary and tertiary structure of the molecule [4]. The disulfide bonds are very important for the tertiary conformation of the proteins and control the biological activity of the biomolecule.

Circular dichroism (CD) in the wavelength range of 250–330 nm has been used to study the behavior of disulfides and their functions in the tertiary structure of proteins theoretically and experimentally [5,6], which gives some information about the screw sense of the unstained disulfide from its dihedral angle through a quadrant rule. There are several methods available for CD spectra analysis of secondary structures of proteins [5,7], such as singular value decomposition (SVD), multiple linear regression (MLR), self-consistent (SELCON), but they cannot be used for the CD spectra analysis of the tertiary structures. Here a singular value decomposition least square (SVDLS) method which combined SVD with the least square method (LS) was suggested, and this can be used in not only the CD spectra analysis of the secondary structures but also the tertiary structures.

Electrochemical reduction of disulfides in BSA at the mercury electrode shows a strong adsorption, and the number of reducible disulfides greatly depends on experimental conditions. For example, Cecil and Weizman [8] studied briefly the polarographic reduction of BSA at a dropping mercury electrode (DME). They observed a single wave in pH 1 solution with $E_{1/2}$ at -0.26 V; the current increased with concentration of BSA till reaching a limiting value of 0.02 μA at 2.5 μM. A very small wave was observed at -0.8 V at 30 μM BSA in pH 9.2 solution. They estimated the value of $n = 10.5$ and the diffusion coefficient of 4.49×10^{-7} cm^2 s^{-1} for the reduction process using Ilkovic equation. Kuznetsov and Shumakovich [9] concluded from their study of BSA adsorption at a mercury electrode by a.c. polarography, that BSA was irreversibly adsorbed and reversibly reduced and re-oxidized. Stankovich and Bard [10] studied the electrochemical reduction of BSA at the mercury electrode by cyclic voltammetry and double potential step chronocoulometry in pH 7.4 buffer solution. The proposed mechanism involved the reduction of an adsorbed monolayer of BSA. At short times, three or four disulfide bonds were reduced. The product remained adsorbed and might be re-oxidized. On the coulometric time scale (hours), eight or nine of the total 17 disulfide bonds were broken and an insoluble product which cannot be re-oxidized was formed.

The reduction of disulfides directly influence the tertiary conformation of BSA, which offers a way to study the effect of denaturants on the rigidity of disulfides in proteins [11]. CD spectroelectrochemical method is suitable to investigate the influence of reduction of disulfides on the conformations of protein, but the strong adsorption of BSA molecule on a metal electrode surface may also induce the conformational changes as well. To reduce the influence of BSA adsorption, a glassy carbon(GC) electrode with a mirror finish surface may be a suitable choice. In this paper, in-situ CD spectroelectrochemistry with a long optical path thin layer cell (LOPTLC) was used to investigate the redox reactions of disulfides in BSA at GC electrode and their influence on the secondary and tertiary conformations of BSA.

2. Experimental

2.1. Instruments

Spectroelectrochemical experiments were car-

ried out in a LOPTLC made in our laboratory [12] with a AVIV 62A DS circular dichroism spectrometer (made in USA) for UV spectrum measurement and a PAR-370 electrochemical instrument (made in USA) for electrochemical operation. The LOPTLC is of 10.0 mm optical path length and 0.02 mm thickness of thin layer. A piece of GC(8 mm × 8 mm) inserted in one wall of the thin layer served as the working electrode, a platinum wire as an auxiliary electrode and Ag/AgCl (KCl saturated) electrode as reference electrode. All potentials were reported with respect to this reference electrode. The incidence light passed through the thin layer being parallel to the working electrode, so that only the biomolecule in the solution layer could be monitored during the experiments.

2.2. Reagents and solutions

BSA, purchased from Sigma chemical company (USA), was used without further purification. A 0.125 mg/ml BSA in 10 mM phosphate buffer solution (pH 7.21) was prepared and saturated with N_2 prior to use. All the other reagents were analytical pure. All the solution were prepared with double distilled water.

2.3. Data analysis method

*S*ingular value decomposition least square method. Singular value decomposition least square method (SVDLS) was developed for the data analysis of multiple CD spectra. The principle is briefly described.

$n \times m$ absorbance are measured at n wavelength for m samples composed of k components in the 1-cm optical path length of a cuvette. If the absorbance follow Lambert–Beer's law and additional property, each of the absorbance can be expressed as,

$$a_{i,j} = \Sigma E_{i,l} c_{j,l} \quad (i = 1,2,\ldots n;\ j = 1,2,\ldots m;\ l = 1,2,\ldots k) \tag{1}$$

where $E_{i,l}$ are the absorption coefficient of sample l at wavelength i, $c_{j,l}$ are the concentrations of component j in sample l. The absorbance can be expressed as an absorption matrix and indicated as a bold uppercase letter, $\mathbf{A}_{n\times m}$,

$$\mathbf{A}_{n\times m} = \mathbf{E}_{n\times k}\mathbf{C}_{k\times m} \tag{2}$$

According to SVD [13], **A** can be decomposed into three multiplied matrices;

$$\mathbf{A} = \mathbf{USV} \tag{3}$$

where **U** and **V** are the $n \times n$ and $m \times m$ orthogonal matrices, respectively, and **S** is an $n \times m$ matrix whose off-diagonal entries are all 0 and whose diagonal elements are in a descending order,

$$s_1 \geq s_2 \geq \ldots \geq s_m \geq 0 \tag{4}$$

the s_j^2 are the eigenvalues of matrix **A**. If the samples are composed of k components, the first k eigenvalues are called the principal factors ($k < m$), which can be used to describe the main properties of matrix **A**, while the other eigenvalues are responsible for measurement error. The multiplied matrix of submatrices from the first k rows of **U**, first k columns of **S** and **V**, respectively, is called abstract absorption matrix, and indicated as, $\overline{\mathbf{A}}_{n\times m}$,

$$\overline{\mathbf{A}}_{n\times m} = \overline{\mathbf{E}}_{n\times k}\overline{\mathbf{C}}_{k\times m} \tag{5}$$

where $\overline{\mathbf{E}}_{n\times k} = \mathbf{U}_{n\times k}\mathbf{S}_{k\times k}$, is called abstract absorption coefficient matrix, $\overline{\mathbf{C}}_{k\times m}$ is called abstract concentration matrix. The standard deviation (S.D.) between **A** and $\overline{\mathbf{A}}$ is,

$$\text{S.D.} = \Sigma\Sigma[(\mathbf{A} - \overline{\mathbf{A}})/(n \times m - k)]^{1/2} \tag{6}$$

To transfer the matrices $\overline{\mathbf{E}}$ and $\overline{\mathbf{C}}$ into **E** and **C**, the least square method is used. According to least square method [14],

$$\overline{\mathbf{E}} = \mathbf{A}\overline{\mathbf{C}}^t\left(\underline{\mathbf{CC}}^t\right)^{-1} \tag{7}$$

$$\overline{\mathbf{C}} = (\overline{\mathbf{E}}^t\overline{\mathbf{E}})^{-1}\overline{\mathbf{E}}^t\mathbf{A} \tag{8}$$

and concentration for each component is a non-negative quantity,

$$\overline{\mathbf{C}} \geq 0 \tag{9}$$

According to Eq. (9), setting the values of negative elements in $\overline{\mathbf{C}}$ to 0, and using Eqs. (7) and (8) for several times until the S.D. becomes stable, then $\overline{\mathbf{E}} = \mathbf{E}$ and $\overline{\mathbf{C}} = \mathbf{C}$.

2.3.1. Double logarithmic analysis [15]

If an irreversible electro-reduction reaction occurs in a thin layer solution, the concentration of reactant, $c(E)$, has an exponential relationship with applied potential,

$$c(E) = c^* \exp(-ka/d) \tag{10}$$

where c^* is the initial concentration of the reactant, a is the electrode surface, d is the thickness of the thin layer, k is heterogeneous electron transfer rate constant, which can be expressed as,

$$k = k^0 \exp[-\alpha nF(E - E^0)/RT] \tag{11}$$

where k^0 is standard heterogeneous electron transfer rate constant, αn is the product of electron transfer coefficient and number of electrons, E and E^0 are the applied potential and apparent formal potential, respectively. The other symbols have their common meanings. The normalized concentration of the reactant is,

$$\bar{c}(E) = c(E)/c^* \tag{12}$$

Taking the double logarithm of $1/\bar{c}(E)$, a linear relation, called double logarithmic relation, is obtained,

$$\mathrm{LnLn}[1/\bar{c}(E)] = \ln(k^0 a/d) - \alpha nFE/RT + \alpha nFE^0/RT \tag{13}$$

For EC mechanism [15],

$$c(E) = \{1 - \exp[-k^0 \exp(-\alpha nF(E - E^0)/RT)]/(K_c + 1)\} \tag{14}$$

E^0 can be obtained from the peak position of differential curve of $\bar{c}(E) - E$ with respect to E. αn and k can be obtained from the slope and intercept of the double logarithmic line.

3. Results and discussion

3.1. Electrochemical reduction of BSA

The cyclic voltammograms of 0.45 mg/ml BSA in phosphate buffer solution (pH 7.21) containing 0.20 M KNO_3 at GC electrode at 50 mV s^{-1} are shown in Fig. 1. BSA gives a small but broadened irreversible reduction peak near -0.7 V, with half-wave potential of -0.65 V (vs. Ag/AgCl). This peak corresponds to the reduction of disulfide groups [10]. There are no redox peaks responsible for the adsorbed BSA molecules as obtained at mercury electrode [9,10].

BSA (0.125 mg/ml) in pH 7.21 phosphate buffer solution was put into a LOPTLC for UV-spectroelectrochemical experiment. BSA shows an absorption peak at 278 nm, which corresponds to the tyrosine residues in a close proximity [16]. The disulfide bonds have an absorption peak at 260 nm, but it is too weak to be measured. The absorption peak at 278 nm increases with applied

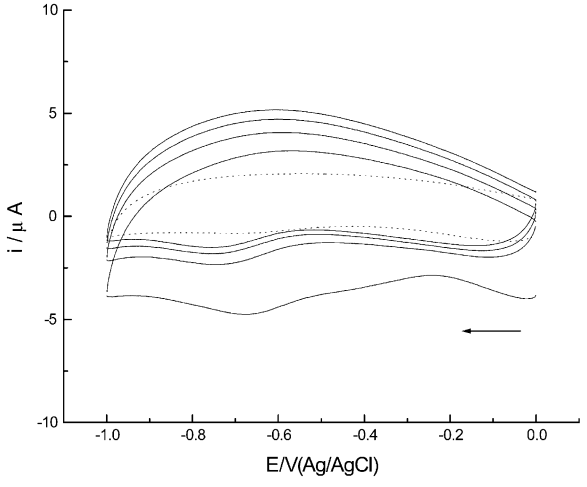

Fig. 1. Cyclic voltammogram of BSA. BSA (0.45 mg/ml) in pH 7.12 phosphate buffer solution on GC electrode. Sweep rate: 50 mV s^{-1}; (---) line: background.

potential shifting to negative direction as shown in Fig. 2. This behavior implies that the reduction of disulfide bonds breaks the links between domains, changes the environment of tyrosine residue resulting in the increase in absorbance. The plot of absorbance at 278 nm against applied potential shows a sigmoid curve (not shown here). The differential curve of the absorbance–potential plot gives a one-peak curve, as shown in Fig. 3b. From the peak position, the apparent formal potential, $E^{0'} = -0.65$ V, is estimated. The double logarithmic plot of the spectroelectrochemical data is an oblique line crossed with a line approximately parallel to the E axis as shown in Fig. 3a. This type of double logarithmic plot suggests that the electrochemical reduction of BSA corresponds to an EC mechanism [15]. The product of the number of electron transferred and electron transfer coefficient, $\alpha n = 0.15$, is estimated from the slope of the oblique line. Using $E^{0'}$, αn and experimental conditions in normal non-linear regression for EC mechanism, the standard heterogeneous electron transfer rate constant, $k^0 = (2.84 \pm 0.14) \times 10^{-5}$ cm s^{-1}, and chemical reaction equilibrium constant, $K_c = 5.13 \pm 0.12$, are obtained with total standard deviation of 0.0504.

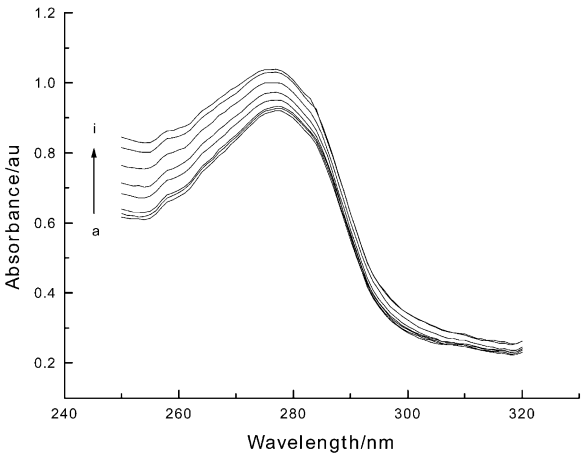

Fig. 2. UV-spectra of BSA with different applied potentials. BSA (0.125 mg/ml) in pH 7.12 phosphate buffer solution. Time interval: 6 min. Applied potential: (a) −0.20; (b) −0.30; (c) −0.40; (d) −0.50; (e) −0.60; (f) −0.65; (g) −0.70; (h) −0.80; (i) −0.90 V.

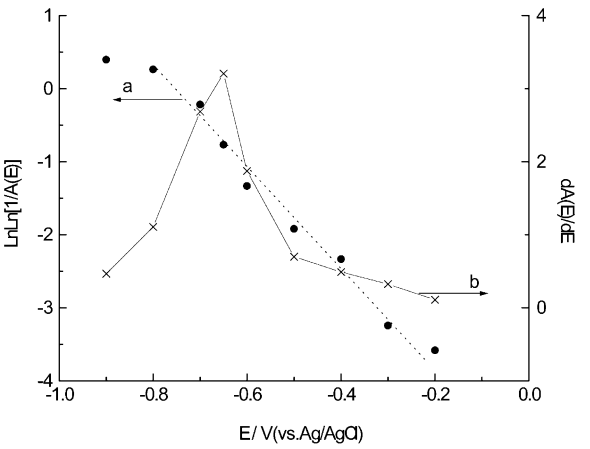

Fig. 3. Double logarithmic plots of $[1/A(E)]$ for BSA. (a) Double logarithmic curve; (b) differential curve. Experimental conditions are the same as those in Fig. 2.

According to the peak current, i_p, expression of an irreversible system [17],

$$i_p = 0.227\ nFac^* k^0 \exp \times \left[-\alpha nF/RT\left(E_p - E^{0'}\right)\right] \quad (15)$$

where n is the number of electrons transferred, a is the area of electrode surface, c^* is the initial concentration of BSA, E_p is the peak potential, other symbols have their common meanings. The area of electrode surface is 0.053 cm^2. i_p ($= 1.87 \times 10^{-6}$ A), c^* ($= 6.52 \times 10^{-6}$ mol/ml), E_p ($= -0.663$ V) can be obtained from Fig. 1. Taking k^0, αn, $E^{0'}$ and other items into Eq. (15), the number of electrons transferred, $n = 8.08$, was obtained. During the electrochemical reduction, each disulfide has two electrons transferred, so there are four disulfides taking part in the electrochemical reduction.

3.2. The tertiary structure changes in electrochemical reduction of BSA

BSA (1.34 mg/ml) in pH 7.12 phosphate buffer solution without other supporting electrolyte was put into a LOPTLC for CD spectroelectrochemical experiment. At different applied potentials,

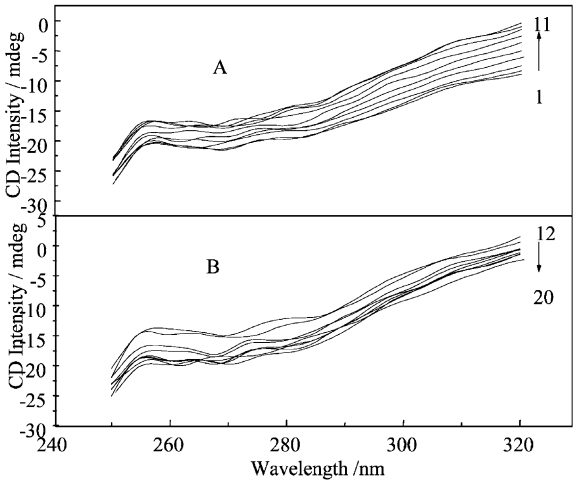

Fig. 4. CD spectra of BSA with different applied potentials in near UV region. BSA (0.125 mg/ml) in pH 7.12 phosphate buffer solution in a LOPTLC. Applied potential: 1, open circuit; 2–11, from 0.0 V to −1.0 V step −0.1 V; 12–20, from −0.9 V to 0.0 V step 0.10 V.

BSA shows a broad CD band from 290 to 260 nm, which decreases with applied potential shifting to −1.0 V from −0.1 V and increases with applied potential returning to 0.0 V from −1.0 V as shown in Fig. 4a,b.

The experimental CD spectra were treated by the SVDLS. The two conformations and their CD spectra are obtained with a mirror-image relationship as shown in Fig. 5. One with a totally negative band is associated with a left-handed screw sense of disulfides at the dihedral angles greater than 90° (Fig. 5, solid line). The other bent to positive direction is associated with a right-handed screw sense at the dihedral angles less than 90° (Fig. 5, dotted line). The fractional distribution of the two conformations with applied potential show a very good mirror-image relation with approximately 0.5 fraction at open circuit for both conformations as shown in Fig. 6. The fractional distribution were normalized with respect to the changes of CD spectra, so monitored only the conformational changes related to the four disulfides taking part in the electrochemical reduction. The 0.5 fraction for both conformations at open circuit imply that 16 disulfides forming eight pairs, each of the latter are

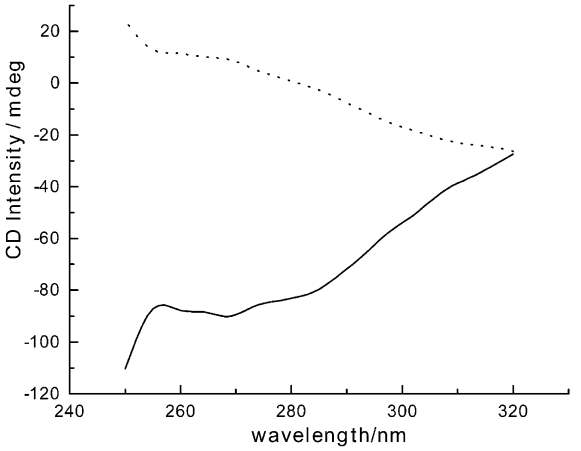

Fig. 5. CD spectra of two components in BSA obtained by SVD analysis. (· · ·) right-handed (—) left-handed.

composed of one with left-handed conformation and one with right-handed conformation. Similarly, two of the four disulfides taking part in the reduction are left-handed and the other two are right-handed. This result is very similar to the previous results that disulfide bonds are likely to form an ensemble with more than two disulfides [18,19]. In the native BSA molecule, 16 of 17 disulfides are composed of eight two-disulfides linkages in eight places and bring the protein chain into close proximity [3].

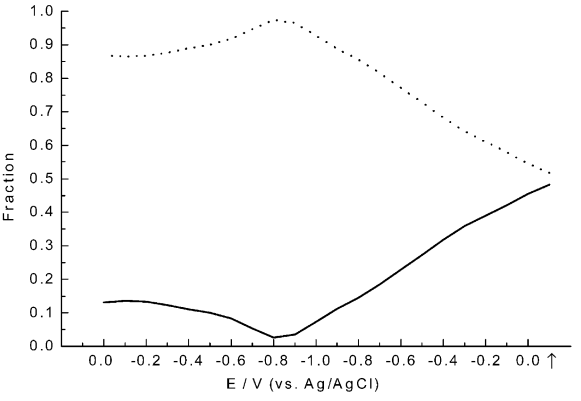

Fig. 6. Distribution curves of two components in BSA with applied potential. (· · ·) right-handed; (—) left-handed. ↑ indicates open circuit case.

When applied potential shifting to negative direction, the distribution curve of the left-handed conformation decreases, while the right-handed conformation curve increases. The maximum conversion rate occurs at the formal potential of BSA (-0.65 V), which implies that the conformation transition is driven by the reduction of disulfides. The reduction of disulfides breaks the disulfide pairs as well as the disulfide bonds. As a result, the disulfide bonds with left-handed conformation (dihedral angles $> 90°$) changed to the right-handed conformation (dihedral angles $< 90°$).

When the applied potential returning from -1.00 V to 0.0 V, some of the reduced disulfides are re-oxidized, and disulfide bonds can be reformed again, so the fraction of the left-handed conformation increases, while one of the right-handed conformation decreases. However, only 25% of the reduced disulfide bonds are recovered, and both of the distribution curves do not return to the original points (distribution fraction is 0.5). This result implies that after reduction the bridges between domains are broken, most of the –S groups go away from each other, where the protein is in a loose state, so that even the potential returning to the oxidation stage, some of the reduced disulfides can not be re-oxidized into disulfide bonds again. It means that the sulfide bonds in reduction are partly irreversible in structure.

4. Conclusions

As a conclusion, the electrochemical reduction process of disulfides in BSA was studied by CD and UV spectroelectrochemical and cyclic voltammetric methods followed by the data analysis of SVDLS. The results showed that the redox reactions of disulfides in BSA resulted in the conformational changes, which greatly influenced the electrochemical activity. The main points of this study were summarized as follows.

1. Under the experimental conditions, only two pairs of the disulfides reduced with an irreversible peak at -0.70 V, which was partly irreversible in structure.
2. The reduction induced the conformational transition of disulfides from left-handed to the right-handed.
3. UV spectroelectrochemical measurements and double logarithmic plot proved to be suitable tools for the extraction of thermodynamic and kinetic information of the irreversible electrochemical reaction, which gives not only the information of mechanism but also the parameters of the process.
4. CD spectroelectrochemical measurement and SVDLS data treatment were suitable for dealing with the tertiary conformational transitions of proteins in an electrochemical process, which gives the number of components, CD spectrum of each component and its distribution fraction with applied potential.

References

[1] R. Cecil, Intramolecular bonds in proteins, I. The role of sulfur in proteins, in: H. Neurath (Ed.), The Proteins, 1, second ed, Academic Press, New York, 1963, pp. 379–476.

[2] I.M. Kolthoff, A. Anastasi, B.H. Tan, Reactivity sulfhydryl and disulfide in proteins. II. Reactive disulfide as related to viscosity and optical rotation in denatured bovine serum albumin, J. Am. Chem. Soc. 80 (1958) 3235–3240.

[3] P.Q. Behrens, A.M. Spiekerman, J.R. Brown, Structure of human albumin, Fed. Proc. 34 (1975) 591.

[4] P. Callaghan, N.H. Martin, The relation of rotatory dispersion behavior of human serum albumin to its configuration, Biochem. J. 83 (1962) 144–151.

[5] R.W. Woody, A.K Dunker, Aromatic and cystine side chain circular dichroism in proteins, in: G.D. Fasman (Ed.), Circular Dichroism and the Conformational Analysis of Biomolecules, Plenum Press, New York, 1996, pp. 112–113.

[6] C. Dieerassi, H. Wolf, E. Bunneberg, Optical rotatory dispersion studies. LXXVIII. Comparative studies of circular dichroism and rotatory dispersion curve. Some observations on sulfur-containing chromophores, J. Am. Chem. Soc. 84 (1962) 4552–4561.

[7] N.J. Greenfield, Methods to estimate the conformation of proteins and polypeptides from circular dichroism data, Anal. Biochem. 235 (1996) 1–10.

[8] R. Cecil, P.D.J. Weitzman, The electroreduction of the disulphide bonds of insulin and other proteins, Biochem. J. 93 (1964) 1–11.

[9] B.A. Kuznetsov, G.P. Shumakovich, Mechanism of the appearances of two stages of protein polarographic wave, Dokl. Akad. Nauk SSSR 210 (1973) 1223–1226 (in Russian).

[10] M.T. Stankovich, A.J. Bard, The electrochemistry of proteins and related substances Part III. Bovine serum albumin, J. Electroanal. Chem. 86 (1978) 189–199.

[11] S. Beychok, Circular dichroism of biological macromolecules, circular dichroism spectra of proteins and nucleic acids provide insights into solution conformations, Science 154 (1966) 1288–1299.

[12] Y. Zhu, G. Cheng, S. Dong, A simple UV-Visible, circular dichroism thin-layer spectroelectrochemical cell with long optical path, Chem. J. Chin. Univ. 12 (1991) 1588–1591.

[13] W.C. Johnson, Jr., Circular dichroism and its empirical application to biopolymers, in: G. Glick (Ed.), Methods of Biochemical Analysis, 31, Wiley, New York, 1985, pp. 61–163.

[14] S.J. Leon, The singular value decomposition, Linear Algebra with Applications, second ed, Macmillan, New York, 1986, pp. 343–376.

[15] Y. Zhu, G. Cheng, S. Dong, A double logarithmic method in thin-layer spectroelectrochemistry for distinction among mechanisms of an irreversible electrochemical process, J. Electrochem. Soc. 146 (1999) 3000–3003.

[16] A.N. Glazer, E.L. Smith, Studies on the ultraviolet difference spectra of proteins and polypeptides, J. Biol. Chem. 236 (1961) 2942–2947.

[17] A.J. Bard, L.R. Faulkner, Electrochemical Methods, Fundamentals and Applications, Wiley, New York, 1980, pp. 222–224.

[18] J. Deisenhofer, O. Epp, K. Miki, R. Huber, H. Michel, Structure of the protein subunits in the photosynthetic reaction centre of *Rhodopseudomonas viridis* at 3 Å resolution, Nature 318 (1985) 618–624.

[19] M.J. Volles, X. Xu, H.A. Scheraga, Distribution of disulfide in the two-disulfide intermediates in the regeneration of bovine pancreatic robonuclease A: further insights into folding process, Biochemistry 38 (1999) 7284–7293.