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Bioelectrochemistry 64 (2004) 33–37

Bioelectrochemistry

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A study on bovine erythocyte superoxide dismutase by controlled potential electrolysis and Raman spectra

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Received 26 August 2003; received in revised form 9 January 2004; accepted 30 January 2004
Available online 9 April 2004

Abstract

The electrochemical behavior of Cu_2Zn_2SOD on mercury electrodes was studied by controlled potential electrolysis. By comparison of UV, Raman spectra and activity of Cu_2Zn_2SOD before electrochemical reduction and after re-oxidation, it is proved that the conformation and activity are not changed.

Keywords: Cu_2Zn_2SOD; Electrochemical behavior; Controlled potential electrolysis

1. Introduction

The electrochemical behavior of proteins on mercury electrodes has been studied for many years. Many papers and several reviews on the adsorption of proteins on mercury electrodes have been reported [1–4], because the studies are involved in the developments of adsorptive stripping methods [5] and in the designing of new molecular devices [6]. Copper–Zinc superoxide dismutase (Cu_2Zn_2SOD or SOD) is an important metal enzyme with defense functions [7], it consists of two identical subunits, each containing one copper(II) and one zinc(II) which are connected by histidine imidazolate bridges. The catalytic reaction occurs at the site of copper, zinc plays a role of stabilizing protein structure. Only a few papers concerning the electrochemical behavior of Cu_2Zn_2SOD on mercury electrodes were reported until now [8]. Recently we reported the abnormal electrochemical behavior of Cu_2Zn_2SOD on mercury electrodes [9]. In general, redox proteins hardly show a defined electrochemical response in the absence of mediators (or promotors) [10]. But in the cyclic voltammetry (CV) or direct current polarogram of Cu_2Zn_2SOD, two pairs of well-defined redox adsorbed peaks were obtained on hanging mercury drop electrodes (HMDE) or dropping mercury electrodes as working electrodes. Two pairs of peaks are attributed to the redox peaks of copper(II) and zinc(II), respectively, and not to those of disulfide bonds of proteins as indicated by other authors [11].

In this paper, the electrochemical behavior of Cu_2Zn_2SOD on mercury electrodes was investigated further by controlled potential electrolysis. Before electrochemical reduction of SOD and after re-oxidation, the following three experiments were carried out. (1) The activity of Cu_2Zn_2SOD was assayed. (2) The absorbances of Cu_2Zn_2SOD solution at 280 and 680nm were measured. (3) Raman spectra of lyophilized Cu_2Zn_2SOD were recorded. By comparison of its UV, Raman spectra and enzymatic activity before electrochemical reduction and after re-oxidation, it is proved that the conformation and activity of SOD are not changed after successive electrochemical reduction and re-oxidation. This is one of the reasons why the well-defined CV diagram and polarogram were obtained.

2. Experimental

2.1. Materials

Cu_2Zn_2SOD was prepared from bovine blood by methods of previous authors [9]. The raw extractive of BESOD (bovine erythrocyte superoxide dismutase) was purified by DEAE-cellulose 32 and Sephadex G 50

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doi:10.1016/j.bioelechem.2004.01.005

successively. The purified BESOD had only one homogeneous and clear band in polyacrylamide gel electrophoresis. The molecular weight of BESOD was measured to be 32,800 by SDS-polyacrylamide gel electrophoresis, its Cu and Zn contents were measured by inductively coupled plasma spectrometry to be 0.398% and 0.402%, respectively, which is consistent with the calculated values of 0.387% and 0.399%. The specific activity was measured by the xanthine oxidase method to be 6000 unit mg^{-1}. The parameters of EPR and peak values of UV spectra were in agreement with those in previous reports [9].

For investigation of Raman spectra of deuterated SOD, the sample was prepared by the following procedure: BESOD (20–30mg) was dissolved in deuterated phosphate buffer solution (pD = 7.0), the solution was stored at 4 °C for 6–8 h for attaining the exchange equilibrium between deuterons of amide and protons. After dialyzing phosphate, the resulting solution was lyophilized to powder. All chemical regents were of A.R. grade and were recrystallized in twice-distilled water before use. The ionic strength of the solutions was kept constant by using KCl.

2.2. Electrochemical measurements

All electrochemical measurements were carried out on an EG and G PARC 270 electrochemical analytical system. In CV experiments, a M303 hanging mercury drop electrode (HMDE) as working electrode, a saturated calomel electrode (SCE) as reference electrode, and a platinum coil wire as auxiliary electrode were used. The potentials were measured vs. the saturated calomel electrode. The *i*R compensation of the cell was made by the apparatus. Each measurement was repeated several times and the redox potentials were found to be reproducible within ± 5 mV. In the cyclic voltammetric experiments, the blank solution was added to 10 cm^3 cell, and then deaerated with a slow stream of super-pure nitrogen for ca. 15 min at 25 °C, a weighted sample of SOD was added to the cell to a predetermined concentration, and deaeration was continued for 10–15 min before applying the voltage. The formal or redox half-wave potentials $E_{1/2}$ were obtained as $(E_{pa} + E_{pc})/2$.

In the controlled potential electrochemical experiments [12], a mercury pool with area 1.54 cm^2 was used as working electrode. About 2 ml of 0.05 mol dm^{-3} phosphate buffer solution (pH = 7.4) containing BESOD 4.70 mg (7.31×10^{-5} mol dm^{-3}) with specific activity 6125 unit mg^{-1} and KCl (0.1 mol dm^{-3}) was added to the cell. Electrolysis was carried out under an argon atmosphere at − 0.8 V for 6–7 min for complete reduction of copper(II) in BESOD. After complete reduction of Cu(II) to Cu(I), the electrolytic current decreased to a constant value, afterwards the applied voltage was changed to − 0.3 V for re-oxidation of Cu(I). The re-oxidation was applieed for 6–7 min to reach complete oxidation of Cu(I) in BESOD. In order to check the behavior of BESOD on a mercury electrode, before electrochemical reduction and after re-oxidation, the following three experiments were carried out. (1) The activity of BESOD in the solution was assayed. (2) The absorbances of the solution at 280 and 680 nm were measured with a Shimazdu UV-3100 spectrophotometer. (3) Raman spectra of lyophilized BESOD from H_2O solution and D_2O solution were measured respectively with a Bruker RFS100 Raman spectrometer.

3. Results and discussion

3.1. Activity and spectral parameter

Controlled potential electrolysis was used to check the behavior of SOD on mercury electrode. During electrochemical reduction of BESOD the current *i* decayed exponentially with time *t* as expected, and *i* remained essentially constant after about 100 s. The total amount of charge *Q* (0.0282C) consumed for the reduction of BESOD was obtained by plotting log *i* vs. *t* (Fig. 1) which corresponds to 4.8 mg reduced BESOD (yield 102%). This indicated that BESOD in solution was reduced completely, 102% reduction might be due to experimental error. In Table 1 the absorbance ratios A_{280}/A_{680}, absorbance coefficients ε and activities of BESOD are shown. From Table 1, it is seen that after suffering successive electrochemical reduction and re-oxidation, activities of BESOD were basically unchanged and the spectral parameters were close to the results of previous authors [10,13,14].

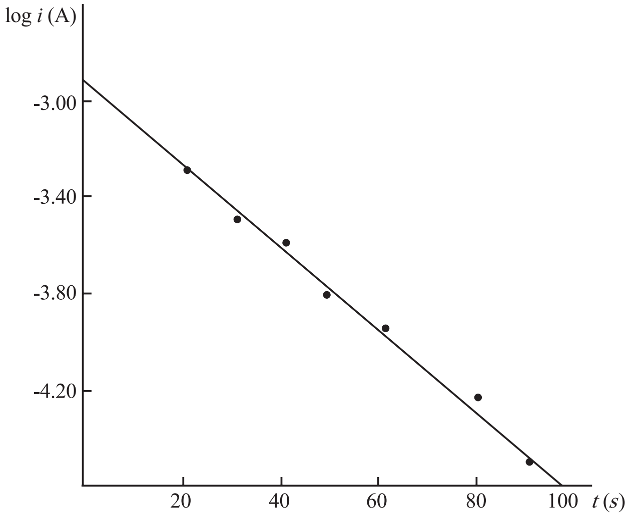


Fig. 1. Dependence of log *i* on *t* for controlled potential electrolysis at − 0.8 V, pH = 7.4 (*i* denotes measured minus background current) 7.16×10^{-5} mol dm^{-3} BESOD in phosphate buffer solution.

Table 1
Electronic spectral parameters and activity of BESOD before electrochemical reduction and after re-oxidation

Electrolysis	ε_λ ($\text{mol}^{-1}\ \text{dm}^3\text{cm}^{-1}$) 280 nm	680 nm	A_{280}/A_{680}	Specific activities (unit/mg)
Before	9989	315	31.7	6285
After	10,109	314	32.2	6273
Literature	9900 [13,14]	300 [13] 310 [14]	33 [10,13]	

Concentration: 7×10^{-5} mol dm^{-3}. pH = 7.4.

3.2. Raman spectra

Raman spectra can be used for determining protein structures because conformational changes are accompanied by the changes of Raman frequency and intensity [15]. Raman spectra of lyophilized SOD before electrochemical reduction and after re-oxidation, respectively, are shown in Fig. 2. The spectrum of SOD before electrochemical reduction is similar to that after re-oxidation. The major bands of SOD (Table 2) are in good agreement with those reported previously [16]. Amide bands of proteins are conformationally sensitive and hence suitable for structural determinations. The relative intensities and vibration frequencies of amide I, II, and III bands were used to determine the conformational contents in lyophilized SOD as indicated by Lippert [17]. The fractions of α-helix (f_α), β-helix (f_β) and random structures (f_r) in SOD were calculated by the following equations:

$$C^{\text{SOD}} I_{1240}^{\text{SOD}} = f_\alpha I_{1240}^{\alpha} + f_\beta I_{1240}^{\beta} + f_r I_{1240}^{r} \tag{1}$$

$$C^{\text{SOD}} I_{1632}^{\text{SOD}} = f_\alpha I_{1632}^{\alpha} + f_\beta I_{1632}^{\beta} + f_r I_{1632}^{r} \tag{2}$$

$$C^{\text{SOD}} I_{1660}^{\text{SOD}} = f_\alpha I_{1660}^{\alpha} + f_\beta I_{1660}^{\beta} + f_r I_{1660}^{r} \tag{3}$$

$$f_\alpha + f_\beta + f_r = 1.0 \tag{4}$$

Here I_{1240}^{SOD} denotes the spectral height of lyophilized SOD from aqueous solution at 1240 cm^{-1} relative to the spectral height of the methylene bending mode at 1448 cm^{-1}, i.e. a relative intensity. I_{1632}^{SOD} and I_{1660}^{SOD} are relative intensities of lyophilized SOD obtained from D_2O solution. I_v^α, I_v^β, and I_v^r denote the relative intensities of a “pure” α-helical protein, a “pure” β-helical protein, and a random structure, respectively, at frequency v. I_v^α was obtained by referring to that of poly-L-lysine in aqueous (or D_2O) solution [17]. C^{SOD} is a scaling constant.

Tables 3 and 4 lists the spectral relative intensities of SOD (I_v^{SOD}) at frequency v = 1240, 1632 and 1660 cm^{-1}, respectively, and its percentages of secondary structures before electrochemical reduction and after re-oxidation, respectively. From the results of Table 4 it is seen that

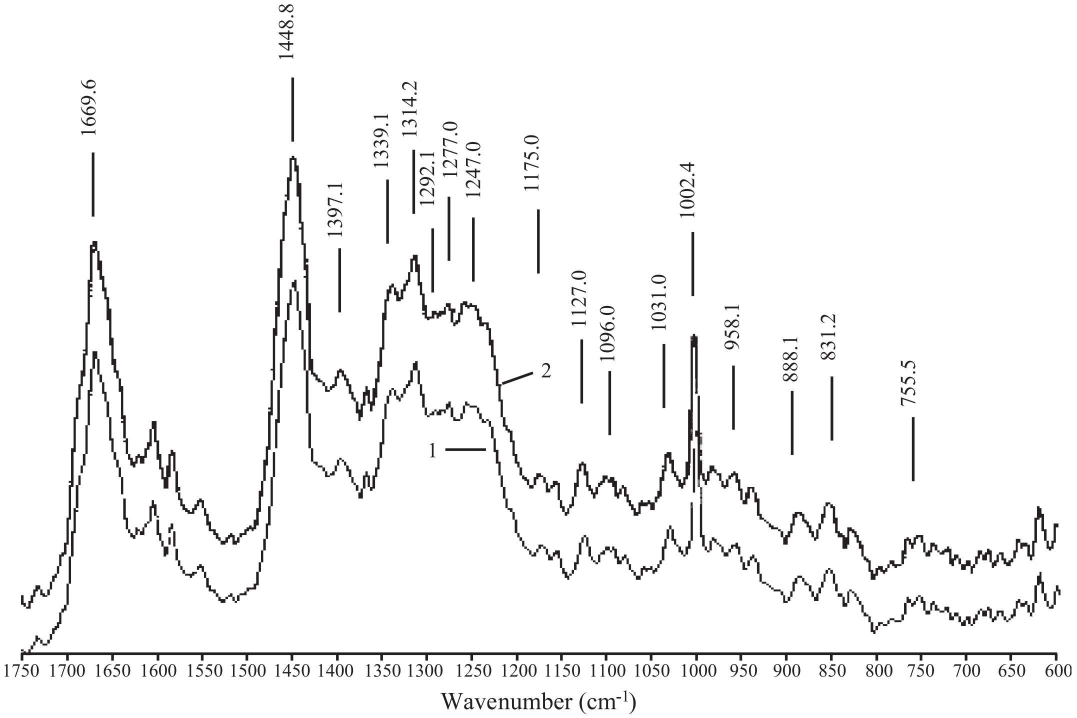


Fig. 2. The Raman spectra of lyophilized BESOD from water solution. (1) before electrochemical reduction; (2) after re-oxidation. Laser power 80 mW.

Table 2
Assignments of Raman bands of BESOD, together with those reported by previous authors

Assignment	Frequency (cm^{-1})	
	This work	[16]
C–C stretch	958.1 (m)	961
phe ring mode	1002.4 (s)	1005
phe	1031.0 (s)	1031
C–C stretch	1096.0 (w)	1096
C–C stretch (*trans*)	1127.0 (m)	1126
phe	1175.0 (m)	1172
amide III	1247.0 (m)	1246
amide III (α)	1277.0 (m)	1265
amide III	1292.1 (w)	1286
$C-H_2$ twist	1314.2 (m)	1317
$C-H_2$ twist	1339.1 (m)	1341
asp, glu CH_2-CH_3 bend	1397.1 (m)	1402
CH_2-CH_3 bend	1448.8 (s)	1451
amide I	1669.6 (s)	1670

before electrolysis the contents of α-helical structure in SOD were low and the contents of β-structures were close to those of random structures, which is in good agreement with the results by X-ray structural determination [18]. After electrochemical re-oxidation, the contents of α-helices were slightly decreased and the contents of β-structures were equal to those of random structures. Raman spectrometry is only an indirect method for the determination of protein structures, hence it cannot be excluded that the experimental data are ambiguous to some extent. Nevertheless, we conclude from the above results that the conformation of SOD was not basically changed after successive electrochemical reduction and re-oxidation.

It is well known that the rate of heterogeneous electron transfer between proteins and electrode surfaces not only depends on the nature of the electrode surface [19] and the proteins [20], but also on the purity of the proteins [21,22]. The cyclic voltammogram of pure BESOD on HMDE (Fig. 3a) consists of two pairs of well-defined adsorbed redox peaks of Cu(II) and Zn(II). The $E_{1/2}$ values are -0.645 and -1.030 V for the Cu(II) and Zn(II), respectively. When comparing the cyclic voltammogram of BESOD with that of PESOD, it is seen that the potential separations of BESOD ($\Delta E_1 = 70$ mV, $\Delta E_2 = 220$ mV) are larger than those of PESOD ($\Delta E_1 = 63$ mV, $\Delta E_2 = 81$ mV). The adsorptive behavior is similar to that of PESOD, which will be reported elsewhere in detail. When stirring a solution of BESOD for two hours at 30–40 °C, the conformation of SOD was damaged, the activity of SOD decreased by 40–50% as compared with the native SOD, and its CV diagram (Fig. 3b) displayed a deformed curve. In addition, the CV diagram of the crude SOD (Fig. 3c) only displayed a very weak and indistinct current signal because impurities are most likely adsorbed on the mercury electrode which shields the electroactive centers. Moreover, this inactivates the electrode and thus suppress the electron transfer between SOD and the electrode surface.

Table 3
The Raman spectral relative intensities of lyophilized SOD (I_v^{SOD}) before electrochemical reduction and after re-oxidation

v (cm^{-1})	1240		1632[a]		1660[a]	
Electrolysis	Before	After	Before	After	Before	After
I_v^{SOD}	0.704	0.699	0.324	0.311	0.680	0.646

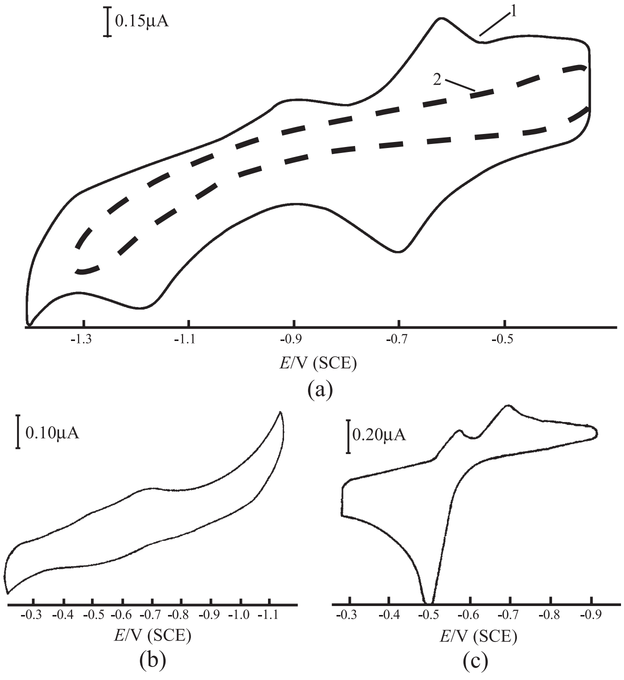
[a] Frequencies obtained from D_2O solution.

Table 4
The percentages of secondary structures of BESOD before electrochemical and after re-oxidation, together with those obtained by X-ray method

Secondary structures	α-Helix		β-Structure		Random structure		Methods
Electrolysis	Before	After	Before	After	Before	After	
Percentages	3.0	0	45.1	50	51.9	50	This work
	5		45		50		X-ray [18]

I_v^{α}, I_v^{β} and I_v^{r} used in the calculation of the percentages of secondary structures were 0.00, 1.20, and 0.60 for $v = 1240$ cm^{-1}; 0.80, 0.72, and 0.08 for $v = 1632$ cm^{-1}; 0.55, 0.88, and 0.78 for $v = 1660$ cm^{-1}, respectively [17].

Controlled-potential electrolysis showed that the spectral parameters, the activity and the conformation of SOD are not changed after successive electrochemical reduction and



(a)

(b)

(c)

Fig. 3. Cyclic voltammogram of BESOD on HMDE. (a) pure SOD: SOD solution (1) and blank solution (2); (b) damaged SOD; (c) crude SOD. Scan rate 0.10 V sec^{-1}, concentration of BESOD is 1.2×10^{-4} mol dm^{-3} in 0.10 mol dm^{-3} KCl solution and 0.05 mol dm^{-3} phosphate buffer at pH = 7.4.

re-oxidation if SOD with high purity is used. Therefore we can obtain a well-defined CV diagram.

Acknowledgements

This work was supported by the National Natural Science Foundation of China.

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