Cyclic voltammetry: A new strategy for the evaluation of oxidative damage to bovine insulin

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Abstract: Research on protein oxidative damage may give insight into the nature of protein functions and pathological conditions. In this work, the oxidative damage of bovine insulin on Au electrode was investigated by cyclic voltammetry (CV). The experimental results show that there are two anodic peaks for the oxidative damage of bovine insulin, which arise from the oxidation of the exposed disulfide bond S—S_{CYS7A,CYS7B}, forming sulfenic acid RSOH (1.20 V, vs. SCE), sulfinic acid RSO₂H and sulfonic acid RSO₃H (1.35 V, vs. SCE). These *in vitro* findings not only demonstrate the applicability of CV in simulating/evaluating the oxidative damage of nonredox proteins but also find two promising candidates (two anodic peaks) for measuring insulin.

Keywords: bovine insulin; oxidative damage; evaluation strategy; cyclic voltammetry

Introduction

The diverse physiological functions of proteins are closely related to their integrated structures.^{1–4} Once oxidative damage occurs in proteins, their corresponding functions reduce or disappear, thereby inducing aging, diabetes, cancer, cardiovascular disease, and other pathological conditions.^{5–7} The inherent correlations between the aforementioned diseases and protein oxidative damage have been widely confirmed and have aroused wide attention.^{1,8–10} Investigation into the oxidative damage mechanism of proteins not only benefits to clarify

protein functions, but also contributes to disease diagnosis, prevention and treatment.^{6,10–12}

In evaluation technologies for oxidative damage to proteins, electrochemical technology is sensitive, accurate, selective, simple, clean, and nondestructive.^{13,14} The direct or indirect electron exchange processes between electroactive components and electrode are similar to the oxidative processes of biomolecules *in vivo*.¹⁴ Therefore, the electrochemical mechanism for the oxidation of proteins is suitable for simulating and explaining the oxidative damage of proteins *in vitro* and *in vivo*. With the development of electrochemical apparatus and the application of modified electrodes¹⁵ and microelectrodes,¹⁶ electrochemical technology has obvious advantages in study of protein oxidative damage.

In previous studies, research mainly focused on redox proteins (iron porphyrin proteins, etc.), and little work was related to the side chain oxidation of amino acids in proteins.^{17–19} Therefore, a thorough investigation on the processes or mechanisms of protein oxidative damage has a great significance. In this article, an important functional protein of bovine insulin was selected as an oxidative target and

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Figure 1. Cyclic voltammograms of phosphate buffer, bovine insulin, and cystine. Conditions: curve A 0.05 mol/L phosphate buffer, curve B 0.1 mmol/L bovine insulin, curve C 0.1 mmol/L cystine; initial potential -0.5 V; scan range -0.5 to 1.6 V; scan rate 100 mV/s; pH = 2.5 ; temperature 20°C . [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

its oxidation mechanism on Au electrode was evaluated by CV.

Results and Discussion

Cyclic voltammetry analysis

The cyclic voltammograms of phosphate buffer and bovine insulin are shown in Figure 1. Phosphate buffer (Fig. 1, curve A) has a pair of redox peaks, the anodic peak is at 1.16 V and the cathodic peak is at 0.69 V. Phosphate cannot be oxidized at such low potentials and both peaks are corresponding to the redox processes of Au electrode. The anodic peak arises from the formation of AuO_x and the cathodic peak corresponds to the reductive process.^{20,21} Compared with curve A, bovine insulin (Fig. 1, curve B) has an increased anodic peak near 1.20 V, but a stable cathodic peak near 0.70 V. So this anodic peak should not only be attributed to the electrochemical oxidation of Au electrode but also be assigned to the oxidation of insulin. It is clear that the new anodic peak near 1.35 V corresponds to another oxidation process of bovine insulin. Thus bovine insulin has two irreversible oxidation processes on Au electrode.

To further investigate the oxidative damage mechanism and determine the damage sites, we scanned all amino acids within insulin under the aforementioned experimental conditions. Specific experimental results (Table I) showed that cystine in bovine insulin was the only potential oxidative site due to its anodic peaks at 1.03 and 1.18 V. The cyclic voltammogram of cystine was also given in Figure 1 curve C. Sample of cystine has a similar cathodic peak to that of phosphate buffer, meaning a stable redox process (a stable anodic peak) for Au electrode. Explanations for the significantly increased anodic peak (near 1.18 V) and the new coming anodic peak (near 1.03 V) are the newly formed products. Under the same conditions, glycine has not been oxidized, which means that carboxyl and amino groups can not be oxidized. Therefore, the oxidative site of cystine is limited to its S–S bond. The oxidation mechanism of cystine has been clarified and it can be oxidized into sulfenic acid in a free manner but hard to be oxidized into sulfinic acid or sulfonic acid.²² So the oxidation of cystine on working electrode can occur by the following mechanisms:

Scheme 1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

By comparing structures of bovine insulin and cystine, it can be concluded that disulfide bonds (S–S, part of cystine) in insulin are the candidates of oxidative damage sites. At the same time, the oxidative damage processes of insulin and cystine both

Table I. Electrochemical Characteristic of Insulin-Containing Amino Acids

Amino acids (0.1 mmol/l)	Structural groups	Anodic peaks
Cystine	Sulfur containing amino acids	1.03 V, 1.18 V
Phe, Tyr	Aromatic amino acids	None
Gly, Val, Leu, Ile, Glu, Pro, Ala	Aliphatic amino acids	
Lys, Arg, Gln, Asn, His	Amide containing amino acids	
Ser, Thr	Hydroxyl containing amino acids	

The anodic peak of cystine near 1.18 V is much higher than that of phosphate buffer, suggesting new oxidation product formed; the anodic and cathodic peaks of other amino acids are close to that of phosphate buffer, thus these peaks should be attributed to the redox processes of Au electrode.

Conditions: initial potential -0.5 V; scan range -0.5 – 1.6 V; scan rate 100 mV/s; pH = 2.50 ; temperature 20°C .

Scheme 2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

include two irreversible processes. However, due to steric hindrance, the oxidative damage of cystine can be achieved under lower potentials. Thus the oxidative damage mechanism for insulin is as follow:

Bovine insulin has three disulfide bonds (S—SCYS7A-CYS7B, S—SCYS6A-CYS11A, and S—SCYS20A-CYS19B), implying three candidates that can be oxidized in electrochemical processes. The structure and the cystine of bovine insulin are shown in Figure 2. But they have different surface areas (Fig. 2), thus the relative degree of oxidative damage is different. By comparing the solvent accessible surface areas (SASA) of these disulfide bonds within insulin using the GETAREA 1.1 software, the oxidation order can be evaluated. The results show in Table II. From Table II, we can see that S—SCYS6A-CYS11A or S—SCYS20A-CYS19B has small solvent accessible surface areas and can not be oxidized. Thus the damage site within insulin is only S—SCYS7A-CYS7B.

The influences of dissolved oxygen, scan rate, pH, and temperature on the oxidative damage processes

Influence of dissolved oxygen. With vacuum deaeration, the anodic peak potentials shift negatively and their currents increase markedly (Table III), meaning dissolved O_2 has adverse influence on the oxidation process of insulin. A possible explanation for this phenomenon is that dissolved O_2 tends to adhere to the electrode and block the oxidation of insulin and Au electrode.²³ To get reliable and steady results, all samples should be degassed before the scan.

Table II. SASA of Disulfide Bonds in Bovine Insulin

Atoms	S—SCYS7A,CYS7B	S—SCYS6A,CYS11A	S—SCYS20A,CYS19B
SASA of Disulfide bonds ($\text{\AA}^2$)	37.36	0.01	0
Oxidation order	S—SCYS7A,CYS7B > S—SCYS6A,CYS11A $\approx$ S—SCYS20A,CYS19B		

The 3-D structural data of bovine insulin gets from Protein Data Bank and its PDB code is 1B18.

Figure 2. Structure of disulfide bonds in bovine insulin Chain A: Blue; Chain B: Green; Cystines are in the state of ball and stick. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Influence of scan rate. Relations between scan rate and anodic peak currents are in favor of determining the control steps of oxidative damage. The influence of scan rate on bovine insulin anodic peaks is discussed here (Fig. 3).

The currents of anodic peaks and $v^{1/2}$ have a linear relationship. For example, the linear regression curve of anodic peak a is $i_a = -3.47 + 11.96 v^{1/2}$ (i : 10^{-7} A, v : mV/s, $R = 0.9873$) and that of peak b is $i_b = -132.5 + 32.93 v^{1/2}$ (i : 10^{-7} A, v : mV/s, $R = 0.9965$). We also find that there is a linear relationship between peak currents and scan rate. Taking peak a as an example, the linear regression curve is $i_a = 55.1 + 0.53 v$ (i : 10^{-7} A, v : mV/s, $R = 0.9995$). For peak b, the linear regression curve is $i_b = 31.12 + 1.45 v$ (i : 10^{-7} A, v : mV/s, $R = 0.9971$). When these processes are controlled by either diffusion or adsorption, all linear regression curves should go through the origin point. In fact, the x-intercepts and y-intercepts of these curves are not zero. So we can conclude that the oxidative damage of bovine insulin is synchronously controlled by diffusion and adsorption.

The influence of pH. The influence of pH on anodic peaks is shown in Figure 4. The currents of both anodic peaks are stable between 2 and 3.5 and reach the maximum signals near 2.5. The potentials of both peaks are decreased with a higher pH. But due to the influence of electrode oxidation, peak a

Table III. *The Influence of Dissolved Oxygen*

	Potentials/mV		Currents/ 10^{-7} A	
	Peak a	Peak b	Peak a	Peak b
Without vacuum disposal	1090	1250	860	1403
With vacuum disposal	1110	1286	1110	1850
Rate of increase	1.83%	2.89%	24.72%	31.86%

Conditions: Bovine insulin 0.1 mmol/L; initial potential -0.5 V; scan range -0.5 to 1.6 V; scan rate 100 mV/s; pH = 2.50 ; temperature 20°C .

Figure 3. The influence of scan rate. Conditions: Bovine insulin 0.1 mmol/L; initial potential -0.5 V; scan range -0.5 to 1.6 V; pH = 2.5 ; temperature 20°C . [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

has a more pronounced downward trend. So pH 2.5 was chosen as a suitable experimental condition.

These results are due to the structural changes of insulin. When pH is lower than its isoelectric point (5.25–5.35), insulin is positively charged. It is difficult for insulin to migrate to the negatively charged working electrode (positive scan), resulting in the reduction of peak currents. At the same time, with the increase of pH, insulin gradually formed polymers and will hinder the processes of electrochemical oxidation.²⁴

The influence of temperature. Temperature has a complicated influence on the anodic peaks (Fig. 5). When temperature increases from 5 to 45°C , both peaks gradually shift in negative direction and have increased currents. A higher temperature can promote the diffusion of bovine insulin, thus enhancing the oxidative processes on working electrode.¹⁴ This result is consistent with the diffusion-controlled oxidative processes occurring at the working electrode. But when the temperature is lower than 5°C , they have obviously increased currents and decreased

Figure 4. The influence of pH. Conditions: Bovine insulin 0.1 mmol/L; initial potential -0.5 V; scan range -0.5 to 1.6 V; scan rate 100 mV/s; temperature 20°C . [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 5. The influence of temperature. Conditions: Bovine insulin 0.1 mmol/L; initial potential -0.5 V; scan range -0.5 to 1.6 V; scan rate 100 mV/s; pH = 2.5 . [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

potentials. The mainspring for the increased currents is the degeneration of bovine insulin, which can increase the contact area of water and disulfide bonds, finally is in favor of the oxidative damage processes.

In summary, cyclic voltammetry was shown to be well suited for simulating and evaluating the oxidative damage processes of bovine insulin. These processes were compared with that of phosphate buffer and cystine and all related amino acids within insulin. The influences of dissolved oxygen, pH, scan rate, temperature were also investigated in detail. Experimental results prove that the disulfide bonds within bovine insulin are the target of electro-oxidation and the oxidative products are sulfenic acid RSOH , sulfinic acid RSO_2H , and sulfonic acid RSO_3H . But due to steric hindrance, the oxidative damage to insulin requires more stringent conditions than that of cystine (free disulfide). The electrochemical processes are not only controlled by adsorption and diffusion but also obviously affected by dissolved oxygen, pH, and temperature. These new findings will give clues for understanding the mechanisms of oxidative damage to bovine insulin and other nonredox proteins.

Materials and Methods

Reagents and apparatus

Asparagine, arginine, bovine insulin, cystine, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, proline, phenylalanine, serine, tyrosine, threonine, tryptophan, valine (Sinopharm Chemical Reagent Co.); H_2SO_4 , H_3PO_4 (Tianjin Tianda Chemical Reagent Co.); 30% H_2O_2 , Ethanol, (Tianjin Guangcheng Chemical Reagent Co.). All reagents are analytical reagents or biochemical reagents and prepared with ultrapure water. All experiments were carried out on a CHI 802B electrochemistry workstation equipped with a standard tri-electrode

system. And it was composed by a CHI101 Au working electrode, a saturated calomel reference electrode, and a platinum wire counter electrode. The phosphate buffers were prepared using a pHs-3C acidity meter (Shanghai Pengshun Scientific Instrument Co.). Samples were degassed in a 2XZ-0.5 vacuum pump and stored in a vacuum desiccator. An electric-heated thermostatic water bath (Jiangsu Jintan Instrument Co.) was used for temperature control.

Pretreatment of Au electrode

To ensure the reproducibility and stability of experimental results, the pretreatment of working electrode is essential. Before each experiment, the Au electrode was polished with a fine sand paper (0.25 mm) and Al_2O_3 powers (1 μm , 0.3 μm , and 0.05 μm , successively), rinsed with ultrapure water, and dipped into *Piranha* solution for 10 mins. After rinsing with ultrasonic in ultrapure water and ethanol, the electrode was preserved in ethanol.

Experiment method

Series of phosphate buffers were prepared by adding 0.05 mol Na_2HPO_4 into 0.05 mol H_3PO_4 . Quantitative insulin was weighed up and added into phosphate buffers. After deoxidized with a vacuum pump, the samples were scanned in a tri-electrode cell. Idiographic experiment conditions (temperature, pH, scan rate, and scan range) are described in Results and Discussion section.

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