Electrochemistry of Xanthine Oxidase and Its Interaction with Nitric Oxide

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With the help of nanocrystalline TiO₂, the direct electrochemistry of xanthine oxidase (XOD) was achieved and two pairs of redox waves were observed. The interaction between XOD and nitric oxide (NO) was also investigated. The experimental results reveal that NO can be reduced at a XOD-nano TiO₂ film modified electrode. When the NO concentration was low, the reduced product, HNO, would inactivate the protein. However, when the NO concentration was high, HNO would continue to react with NO to form N₂O₂⁻ and N₃O₃⁻, which would not inhibit XOD, and thus the amount of active protein did not decrease any further.

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Introduction

In mammalian cells, xanthine oxidase (XOD) is a metalloflavoprotein that plays a variety of important roles in the metabolism of purine and pyrimidine. It can catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid, respectively, with a concomitant reduction of the oxygen molecule.^{1,2} Although XOD has been isolated from a wide range of organisms, all of these proteins have similar molecular weights and compositions of the redox active sites.^{1,3} Each subunit of XOD contains a molybdenum center, two non-identical [2Fe-2S] clusters, and a flavin adenine dinucleotide (FAD) cofactor.^{4,5} Recently, increasing attention has been devoted to investigating XOD by the electrochemical technique.^{6,7}

Nitric oxide (NO) is an important regulatory molecule that plays essential roles in many physiological and biological processes, including neurotransmission, platelet aggregation, macrophage function, and vasodilation.⁸ NO can regulate and protect some physiological processes when its concentration is low. On the other hand, when the concentration is high, NO deactivates most enzymes and displays toxicity.^{9,10} Studies have revealed that NO at a high concentration reacts with XOD, glucose oxidase and horseradish peroxidase, and inhibits their catalytic activity.⁹⁻¹² *In vivo* NO can be synthesized by nitric oxide synthases (NOSs), which metabolizes arginine to citrulline.^{13,14} It can also be synthesized by XOD under hypoxic conditions, which catalyzes the reduction of nitrite to NO.¹⁵ Therefore, studies on the interaction between XOD and NO have received increasing interest.^{16,17}

The electrochemical technique has been a feasible way to study the interaction between biological molecules.¹⁸⁻²² However, since the electrochemistry of XOD is very difficult to achieve, only a few electrochemical studies on XOD have been reported, not to mention electrochemical studies on the interaction between XOD and NO.

Recently, an increasing number of nano materials have been employed in bioelectrochemical studies. Nanocrystalline TiO₂ is a kind of nanomaterial that possesses good biocompatibility and chemical stability. It has been reported to be a suitable material for the immobilization of heme proteins at an electrode surface, and to enhance their electron-transfer reactivity.²³ In this paper, we report on direct electron transfer of XOD with the help of nanocrystalline TiO₂, and the interaction between XOD and NO.

Materials and Methods

Materials

Cow milk XOD was purchased from Sigma and used directly without further purification. Stock solutions (1 Unit/mL) were kept at a temperature of 4°C. Nanocrystalline TiO₂ powder was obtained from Nanjing Haitai Nano Company with particle size at about 35 nm. Water was purified with a Milli-Q purification system to a specific resistance >16 MΩ cm⁻¹, and was used to prepare all solutions. 0.1 M phosphate buffer solutions (PBS) of various pH levels were prepared by mixing two stock solutions of 0.1 M Na₂HPO₄ and 0.1 M NaH₂PO₄. All other reagents were of analytical grade.

Sample preparation

NO stock solutions were prepared according to the following procedure. NO gas was generated based on the stoichiometric reaction:

The whole process was performed under a nitrogen atmosphere to prevent NO conversion to NO_x in the presence of O₂. NO gas was purified by passing through three separate trap units: two NaOH solutions (3 mol/L) first, and then pure water. Finally, the NO gas was saturated in a 0.1 mol/L phosphate buffer (pH 7.4). The concentration of NO in the saturated buffer is estimated to be 1.8 mmol/L at 25°C.²⁴ NO analyte solutions

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Fig. 1 Cyclic voltammograms in 0.1 M PBS with pH 6.0 obtained at (a) bare PG electrode (b) nanocrystalline TiO_2 alone modified electrode (c) XOD- TiO_2 film modified electrode. The scan rate was 100 mV/s.

Fig. 2 Cyclic voltammograms in 0.1 M PBS with pH 6.0 obtained at a XOD- TiO_2 film modified electrode (solid) and a FAD- TiO_2 film modified electrode (dot). The scan rate was 100 mV/s.

with desired concentrations were freshly diluted from this stock solution. Other reagents were of analytical grade.

Electrode preparation

The substrate pyrolytic graphite (PG) electrode was first polished using rough and fine sand papers. It was then polished to mirror smoothness with alumina (particle size of about 0.05 μm) and water. Finally, the electrode was thoroughly washed with water, and then treated in an ultrasonic bath for about 5 min, with pure water and ethanol, respectively. The electrode was thus able to be ready for use. Prior to the experiments, a test solution was bubbled thoroughly with high-purity nitrogen. Then, a stream of nitrogen was blown gently across the surface of the solution in order to maintain the solution anaerobic condition throughout the experiments.

An XOD/ TiO_2 film was prepared as follows. An aqueous mixture of 10 μL XOD (1 Unit/mL) and TiO_2 (1 mg/mL) with a ratio of 1:1 was dropped onto the surface of a PG electrode. After that, an eppendorf tube was fitted over the electrode for 2 h to ensure that the water evaporated slowly and a more uniform film structure could be formed. The film was then dried overnight at room temperature. Finally, the electrode was thoroughly rinsed with pure water. The modified electrode was kept in a phosphate buffer solution of pH 7.2 at 4°C when not in use.

Electrochemical measurements

Electrochemical experiments were performed with a PARC

Fig. 3 Cyclic voltammograms of a XOD- TiO_2 film modified electrode in PBS with pH 6.0 containing (a) no NO and (b) 1×10^{-4} mol/L NO. The scan rate was 50 mV/s.

283 potentiostat/galvanostat (EG&G, Princeton, NJ), using a three-electrode configuration. A saturated calomel electrode (SCE) and a platinum electrode served as reference and counter electrodes, respectively. Potentials are reported with respect to SCE unless specially stated. A 50 mL reagent bottle was used for the electrolytic cell. A multi-block heater (LAB-LINE Instruments, Inc., USA) was employed to maintain the experimental temperature at $20 \pm 0.5^\circ\text{C}$. The electrochemical results reported here are all reproduced for 5 times. The current of each peak was measured as the distance from the vertex of the peak to the tangent of its baseline. To study the interaction between XOD and NO, the electrode was first kept at a potential of 200 mV for 5 min. The electrode was scanned for 20 cycles in the range from 200 to -1000 mV, so as to keep the height of the original two pair of peaks constant, before NO was added to the buffer.

Results and Discussion

It is usually very difficult to obtain the electrochemical response of XOD. However, with the help of nanocrystalline TiO_2 , the direct electrochemistry of XOD can be achieved. Figure 1 shows cyclic voltammograms (CVs) obtained at a PG electrode modified with XOD- TiO_2 film in a 0.1 M pH 6.0 phosphate buffer solution. It should be noticed that two pairs of peaks can be observed (Peaks I and II). One pair is located at -0.440 V and -0.382 V (Peak I), while the other is at -0.513 V and -0.479 V (Peak II). However, it is clear that no voltammetric response was observed either at a bare PG electrode or a nanocrystalline TiO_2 -alone modified electrode. Such results indicate that these two pairs of peaks are due to the reduction and oxidation of XOD at the electrode. The XOD- TiO_2 film modified PG electrode was fairly stable. No obvious decrease in peak currents was observed after 1 week.

The characteristics of Peak I was examined for the first time. In the potential scan range from 20 to 250 mV/s, both the cathodic and anodic peak currents were proportional to the scan rate (ν), which indicates a characteristic of thin-layer electrochemical behavior.²⁵ At a scan rate range of 50 to 200 mV/s, it could be found that the width at the half height of the first pair of peaks, $W_{1/2}$, remained constant for both the cathodic and anodic peaks, and the ratio of cathodic current to anodic one was close to one, which suggests that XOD undergoes a quasi-reversible redox process when co-modified with TiO_2 on the PG electrode. The cathodic peak potential (E_{plc}) was measured to

Fig. 4 Cyclic voltammograms in 0.1 M PBS with pH 6.0 obtained at A) bare electrode and B) TiO_2 only modified electrode in the absence (solid line) and presence of NO at concentrations of 30 μM (dash line) and 300 μM (dot line). The scan rate was 50 mV/s.

be ca. -0.440 mV, which is consistent with values reported for free FAD and the enzyme-bound FAD.²⁶ An FAD- TiO_2 modified electrode was also used to distinguish these two pairs of peaks, as shown in Fig. 2. Therefore, the peaks, located at -0.440 V and -0.382 V, contributed to the FAD redox reaction.

As for the second pair of peaks (Peak II) with a formal standard potential (E_{pII}^0) of -0.502 mV, the peak separation (ΔE_{pII}) is estimated to be 51.0 mV, which means that the electron transfer number of Peak II is one (theoretical values of $58/n$ for a Nernstian process).²⁷ Also, the value of E_{pII}^0 is close to that of the $[\text{2Fe-2S}]$ II cluster and is much different from that of the $[\text{2Fe-2S}]$ I cluster.²⁸

It is still unknown how the nanocrystalline TiO_2 caused the enzyme to exhibit this electrochemical behavior, and further work should be performed. However, the electrochemical response of XOD has obviously been observed, and the interactions between XOD and NO might possibly be studied by the electrochemical method. As is shown in Fig. 3, in the presence of 1×10^{-4} M NO, a new peak appears at a potential of -732 mV, in the negative direction of the redox pairs of XOD. Notably, no corresponding signal is visible at a bare PG electrode or an electrode modified with nanocrystalline TiO_2 alone under the same condition (Fig. 4).

Cyclic voltammetric measurements were also performed when adding a series of NO concentrations at a scan range from 200 to -1000 mV, as displayed in Figs. 5 and 6. Figure 5 shows that the peaks currents of the FAD cofactor and the $[\text{2Fe-2S}]$ II cluster gradually decrease with an increase of the NO concentration from 1×10^{-5} M to 1×10^{-4} M. On the other hand, parallel experiments performed for investigating the effect of NO on the FAD cofactor alone show that the peak shape of FAD does not change after adding NO into the test solution. Thus, the concentration of NO is negatively correlative to the peak currents of both FAD and the $[\text{2Fe-2S}]$ II cluster, and the molecule numbers of activated protein will decrease with increasing NO concentration, which confirms the previous report that NO can deactivate the enzyme and display toxicity.^{9,10} We suggest that the deactivation effect is due to the reduction product of NO by XOD, HNO , at the electrode surface,¹⁷ and that NO binding and reduction might occur at the molybdenum site. The proposed mechanism for the reduction of NO by XOD can be expressed by the following equations:

More interestingly, as shown in Fig. 6, when the NO concentration increases from 1.2×10^{-4} M to 3.4×10^{-4} M, the

Fig. 5 Part of voltammograms of XOD- TiO_2 film modified electrode in PBS with pH 6.0 containing NO with concentration of 20, 40, 60, 80 and 100 μM . The scan rate was 50 mV/s. The actual scan range was from 200 to -1000 mV.

Fig. 6 Part of voltammograms of a XOD- TiO_2 film modified electrode in PBS with pH 6.0 containing NO with concentrations of 120, 160, 200, 240 and 280 μM . The scan rate was 50 mV/s. The actual scan range was from 200 to -1000 mV. The inset shows the linear calibration curve between the catalytic peak current and the NO concentration.

cathodic peak current of FAD does not change. On the other hand, because the based lines of the cathodic curves of the $[\text{2Fe-2S}]$ II cluster in the voltammograms rise with an increase of the catalytic peak current of NO, the actual peak current of the $[\text{2Fe-2S}]$ II cluster also does not change. Further, a linear relationship between the peak current of NO and its concentration is obtained. Meanwhile, all of these results indicate that in this NO concentration range, the amount of active protein remains constant. A possible explanation might be that although protonated HNO is the majority in the pH 6.0 test solution, NO^- still exists in the solution. As a result, NO^- might react with NO according to the following reactions if there are more NO molecules:

Both N_2O_2^- and N_3O_3^- will not decrease the activity of XOD.²⁹ Therefore, when the NO concentration is very high, HNO , the reduction product of NO, would continue reacting with NO. Consequently, the enzyme is not inactivated.

Conclusion

With the help of nanocrystalline TiO₂, two pairs of peaks of XOD have been obtained corresponding to the FAD cofactor and the [2Fe-2S] II center. Furthermore, the interaction between XOD and NO was studied *via* the electrochemical technique. When the NO concentration was lower than 120 μ mol, an inhibition effect of NO on these two active centers could be detected, based on a decrease of the peak currents. Interestingly, as the concentration of NO increased above this value, NO was progressively reduced without any further enzyme inactivation, which might have been due to the unarmful products at a higher NO concentration.

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