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Study and application of perturbation of peroxynitrite on peroxidase–oxidase oscillation

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Abstract

Peroxynitrite was one of the important reactive oxygen species (ROS) which have been focused for many years. Peroxynitrite is an extremely strong and reactive oxidant which can cause many diseases linked to inflammatory processes and autoimmune diabetes, etc. ROS can significantly react with peroxidase and nicotinamide adanine dinucleotide (NADH) which is linked to numerous biological processes. NADH, NAD and horseradish peroxidase (HRP) were included in peroxidase–oxidase (PO) oscillation, dissolved oxygen was concerned with the oscillation and many ROS intermediates came into being in PO oscillation which was sensitive to the perturbation of ROS. The influence of peroxynitrite ($ONOO^-$) on this oscillation system was investigated. It was found that the oscillation amplitude increased when the system was perturbed with peroxynitrite. There was a linear relationship between the increment ratio in the oscillation amplitude and perturbing peroxynitrite concentration in the range 2.50×10^{-8} to 1.56×10^{-6} mol/L. And further experimental results revealed that amplitude increasing may be caused by the effect of peroxynitrite on HRP. Based on this phenomenon, a highly sensitive method for the determination of peroxynitrite was developed.

Keywords: Peroxynitrite; Peroxidase–oxidase oscillation; Nicotinamide adanine dinucleotide; Horseradish peroxidase

1. Introduction

Reactive oxygen species (ROS) have been implicated as an important causative factor in cell damage, including apoptosis and necrosis. Their proposed actions comprise lipid peroxidation, mitochondrial respiratory chain destruction and protein modifications [1–3]. Recent experiments have fastened on the importance of peroxynitrite, the reaction product of the two reactive species nitric oxide and superoxide [4]. Peroxynitrite ($ONOO^-$) can cross cell membrane freely and is an extremely strong and reactive oxidant [5]. Its relatively slow reactions with a wide variety of biological molecules render it highly toxic. Peroxynitrite causes DNA damage, enzyme inhibition, apoptosis and bacterial toxicity [6]. Cells injury aroused by peroxynitrite is considered to contribute to the pathogenesis of a series of diseases, including inflammatory processes, ischemiareperfusion, septic shock and neurodegenerative processes [7,8] atherosclerosis, rheumatoid arthritis, myocardial dysfunction and autoimmune diabetes [9]. Evaluation of the potentially injuring mechanism of peroxynitrite has proved difficult, due to its short life span (whose half-life is 10–15 ms) and low concentration in vivo [11]. In recent years, many literatures are focused on protein and DNA damage caused by peroxynitrite. However, reports about the influence of this active oxygen specie on the cell respiratory chain was very few. Cell respiratory chain was sensitive to ROS and many ROS were cleared out by the multienzyme system in respiratory chain. Reduced nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide (NAD) are important substances in cell respiratory chain. Each plays a central role in the multienzyme redox system in mitochondrial electron transport chain. It has been shown by numerous studies that cellular redox state especially nicotinamide adanine dinucleotide redox state NADH/NAD level may be a primary control site in numerous biological process. Horseradish peroxidase (HRP) is one of the most important peroxidases in biology, and is involved in the formation of free radical intermediates for the polymerization and cross-linking of cell wall components, for the oxidation of secondary metabolites essential for certain pathogenic defense reactions, and for the regulation of cell growth and differentiation [10]. HRP is

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prototypical of mammalian peroxidases with respect to the oxidation of small substrates. Dissolved oxygen, HRP and NADH different redox states were included in peroxidase–oxidase (PO) oscillation, so that PO oscillation is convenient to investigate the effect of peroxynitrite on the oxidation of NADH.

Complex dynamics have become increasingly important in biochemical and physiological contexts in recent years. Oscillation reaction is a complex dynamic behavior of the non-linear reaction between the substances in the reactor when the system is in the state far from equilibrium. The time and space characteristics in oscillation reaction are similar to the biological oscillation in vivo. PO reaction is an outstanding example of an enzyme-mediated non-linear and non-equilibrium reaction with complex dynamics. The PO reaction entails the oxidation of molecular oxygen catalyzed by HRP with NADH as the electron donor [12]. The overall reaction is as follows:

$$2\mathrm{NADH} + \mathrm{O_2} + 2\mathrm{H^+} \xrightarrow{\mathrm{HRP}} 2\mathrm{NAD^+} + 2\mathrm{H_2O} \quad (1)$$

The non-linear behavior of this reaction is usually realized in a reactor containing HRP, methylene blue (MB) and 2,4-dichlorophenol (DCP). NADH and O_2 are supplied continuously to this reaction mixture from external sources. When the reaction takes place at low pH in the presence of DCP, MB, and with continuous supplies of NADH and O_2, the concentrations of O_2, NADH and various enzyme intermediates oscillate [12]. In the past, many workers have focused on the studies of the theoretical and experimental dynamics of chemical oscillators. Olson and Scheeline [13,14] have reported the theoretical basis for quantitative enzyme determinations by using the PO oscillation reaction. Olsen and co-workers [14–16] studied and compared four different peroxidases as catalyst. The dynamics of the PO oscillation are very sensitive to external perturbation, and the PO oscillator is based on a ubiquitous biochemical, NADH. So it holds great promise and has a broad potential for quantitative analysis.

Reactive oxygen species significantly react with peroxidase, and NADH/NAD linked to rhythm, senescence, cancer and death [17]. Some reactive oxygen species, such as $O_2^{\bullet -}$, $HO_2^{\bullet}$, H_2O_2 intermediates came into being in PO oscillation [18]. PO oscillation has been confirmed to be an extremely sensitive for dynamical perturbation by oxidation reaction and the non-linear and non-equilibrium changes. The continuous-flow stirred tank reactor (CSTR) and analyte pulse perturbation (APP) [19,20] extended the application of oscillation. Oscillatory dynamics may provide a means of regulating the effect of the redox substance in the life, such as radical-initiated polymerization. We have studied several kinds of substances based on a "probe" of PO oscillation, and the antioxidation of these substances has been discussed [21–23]. In present work, the effect of peroxynitrite on PO oscillation was proposed with APP technique based on the dissolved oxygen in PO oscillation monitored with an oxygen electrode. It was found trace peroxynitrite could influence macroscopical dissolved oxygen amplitude of PO oscillation. The amplitude changed with the concentration of peroxynitrite perturbing the oscillation. The effect of peroxynitrite on PO oscillation was discussed with kinetic method. And further experimental results revealed that this amplitude increasing may be caused by the action of peroxynitrite on HRP. Based on this, a new method for the determination of peroxynitrite was developed. An approach of APP was adopted for determining the peroxynitrite. The proposed method permits the determination of peroxynitrite over the range 2.50×10^{-8} to 1.56×10^{-6} mol/L. The resolved oxygen concentration was employed as the detection signal. Being free of poisonous substances and tedious derivation, the proposed method is simple, sensitive and environmental-friendly.

2. Experimental

2.1. Chemicals

Horseradish peroxidase (Sigma 330 U mg^{-1}, RZ 3.0), NADH (Sigma), MB and DCP, sodium acetate (NaAc) and acetate acid (HAc), 35% hydrogen peroxide (H_2O_2), sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), manganese binoxide (MnO_2) and 96% phosphonic acid (H_3PO_4) (Shanghai Chemical Agent) were used. All chemicals used were of analytical reagent purity. Peroxynitrite was prepared according to the document [24]. Before preparing the proxynitrite standard solution, the aliquot was degassed with nitrogen. The product peroxynitrite is determined with UV–vis spectrophotometer UVIKON-941 (Kontron Inc.) (λ_{max} = 302.0 nm) and the concentration of peroxynitrite is found to be approximately $4.00–7.00 \times 10^{-4}$ mol L^{-1}. Aliquots of peroxynitrite are monitored spectrophotometrically to accurately determine the concentration before each experiment. For pH measurement, a TOA Electronics Model PHS-3C precision pH meter (Shanghai, China) is used. Doubly distilled water was used throughout.

2.2. Apparatus and methods

Experiments were performed in a glass vessel equipped with an oxygen electrode, and fitted with a thermostating jacket and a magnetic stirrer bar. A 8 ml mixture of 0.10 mol/L NaAc–HAc buffer (pH 5.1), 6.0×10^{-8} mol/L methylene blue, 3.5×10^{-5} mol/L 2,4-dichlorophenol and 6.0×10^{-7} mol/L horseradish peroxidase was used. The temperature was kept constant at $28 \pm 0.1\,^{\circ}C$, controlled with a Shimadzu TB-85 thermostat bath. 1.4×10^{-2} mol/L NADH solution was infused at a low flow rate through a capillary tube connected to a high precision syringe pump (Kloehn, Model 50300). The inflow rate of NADH solution was between 49 and 60 $\mu L\,h^{-1}$. A controlled gas mixture of atmospheric air and nitrogen corresponding to 1.55% (v/v) oxygen at atmospheric pressure was blown over the liquid surface. Oxygen concentration in the solution was measured with an oxygen electrode (Shanghai) and the signal from the oxygen electrode was displayed by a chromatogram workstation (Elite EC2000, Dalian). The conditions, temperature, the surface area of the gas–liquid interface and the stirring rate were kept constant throughout the experiment.

After the oscillation amplitude and period had stabilized, different concentration samples in the same volume were injected. The change percentage (I%) of the oscillation amplitude following perturbation was used as the measurement parameters to construct the calibration plot. The oscillation amplitude of sample and the blank are denoted by A_p and A_{p0}, respectively ($I\% = (A_p - A_{p0})/A_{p0}$).

2.3. Stopped-flow kinetic study of PO oscillation perturbed by peroxynitrite

Kinetic experiments are performed on an SX18MV-R stopped-flow analyzer (Applied Photophysics, UK). In a typical experiment, one syringe contained HRP, and another syringe contained other substances in the PO oscillation system and the perturbing substance in the stopped-flow analyzer. Both syringes were not degassed. The experimental temperature is controlled with a Shimadzu TB-85 thermostat bath. NADH ultraviolet absorption at 340 nm was chosen as the signals in the kinetic curve. To observe the transformation of HRP, the ultraviolet absorption spectrum scanning of the system commixed with $ONOO^-$ was observed through photodiode array. The 0.1 M, pH 5.1 NaAc–HAc buffer was used to keep pH value and ionic strength of PO oscillation system.

3. Results and discussion

3.1. Typical oscillatory behavior

Various types of oscillatory behavior of the PO reaction can be observed in a semibatch reactor (Fig. 1) under different experimental conditions. With increasing inflow rate of NADH solution, the dynamics changed from simple periodic oscillation, double periodic oscillation, chaotic behavior to damped oscillation (Fig. 2). Phase portrait of the oxygen in $[O_2]$ $(t + r)$ is plotted versus $[O_2]$ (t) too. We chose the simple periodic oscillations for the research object.

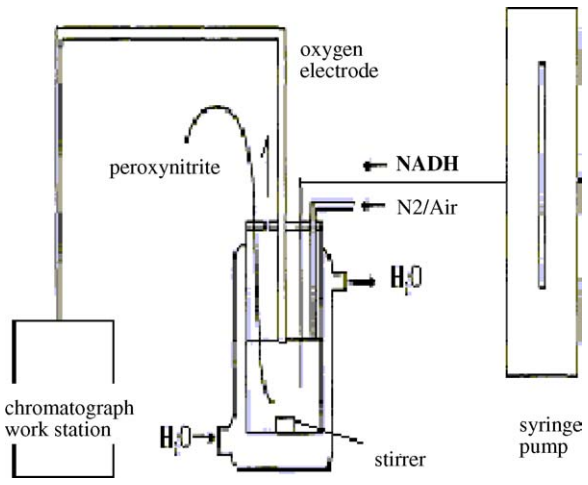

Fig. 1. Schematic diagram of the PO oscillation system. The cuvette's rubberlid contains holes for the oxygen electorde, the inlet of gas and NADH, the gas outlet and injection of peroxynitrite.

3.2. Influence of peroxynitrite on the PO oscillating reaction

To study the effect of peroxynitrite on the oscillatory behavior of the PO oscillation, the APP technique as a tool for analytical determinations in far-from-equilibrium dynamic systems has been used.

Different concentration peroxynitrite in the same valume was taken to perturb this system, which led to oscillation amplitude and period changes. Fig. 3 shows the marked influence of peroxynitrite on the PO oscillation. With the addition of peroxynitrite, there is a rapid increase in the oscillation amplitude and a smaller increase in period for the first cycle and then stable oscillations for the subsequent few cycles. It is evident that the above-mentioned phenomenon becomes more prominent with increasing peroxynitrite concentration. When the peroxynitrite concentration $\geq 1.56 \times 10^{-6}$ mol/L, the oscillation will be inhibited (Fig. 3D). The increased amplitude might result from the influence of peroxynitrite on HRP enzyme intermediates.

3.3. Determination of peroxynitrite

The prepared aliquot without peroxynitrite in the same volume was added into the PO system and no influence was found. It could be concluded that the aliquots of that volume would not influence the PO oscillation. Based on the significant effect of peroxynitrite on the PO oscillation, different concentration peroxynitrite of the same volume was determined. Experiments indicated that there was a linear relationship between the changes ratio in the oscillation amplitude (I%) and peroxynitrite concentration in the range 2.50×10^{-8} to 1.56×10^{-6} mol/L. The calibration data obey the following linear regression equation:

$$I\% = (26.71 \pm 1.77) + (4.58 \pm 0.238) \times [\text{peroxynitrite}] \times 10^7 \quad (r = 0.9946,\ n = 6)$$

3.4. Stopped-flow study of the effect of peroxynitrite on the PO oscillation system

For investigating the influence of peroxynitrite on PO oscillation, the reaction kinetics of PO system perturbed with peroxynitrite was studied through stopped-flow fast scanning spectrophotometer. The rapid kinetics and the initial rate of reaction were observed. Though NADH and O_2 cannot be supplied continuously to the reaction cell in stopped-flow, the dissolved oxygen in the solution was employed and enough NADH was included in the solution, both could satisfy the reaction condition in an oscillation cycle. The concentration of DCP and MB were kept the same to PO oscillation. NADH absorbance at 340 nm was employed as the signal in kinetic curve (Fig. 4), and NAD has not absorption at 340 nm, so that NAD would not influence the observation for NADH. The initial rate for NADH absorbance changing (dA/dt) was used to characterize reaction rate. The absorbance of HRP at 402 nm and the absorbance of intermediates at 420 nm were observed too (Fig. 5). When 3.50×10^{-7} mol/L $ONOO^-$ was introduced into the system, the

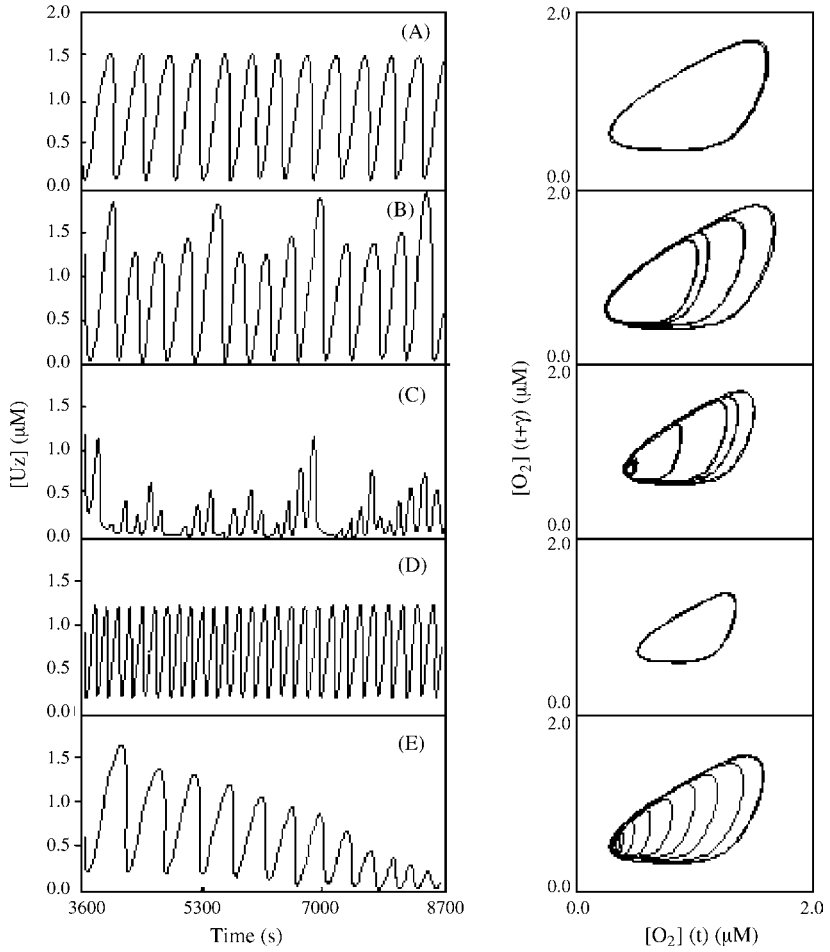

Fig. 2. (Left) Oscillatory dynamics of dissolved oxygen in the PO reaction at increasing inflow rate NADH. (A) 49 μL/h; (B) 55 μL/h; (C) 58 μL/h; (D) 60 μL/h; (E) 65 μL/h. (Right) Phase portrait of the oxygen in (left) $[O_2]$ $(t + r)$ is plotted vs. $[O_2]$ (t), $r = 18$ s.

reaction rate was much faster than the system without $ONOO^-$ in 50 s. NADH absorbance at 340 nm descended more quickly, at the same time HRP and the absorption at 420 nm varied more when peroxynitrite was introduced into the reaction.

In PO oscillation, HRP is oxidized to intermediates Compound I and Compound II which are two of the main HRP intermediates to oxidize NADH [12]. But it has often assumed that the dominant enzyme intermediate in the HRP/NADH system

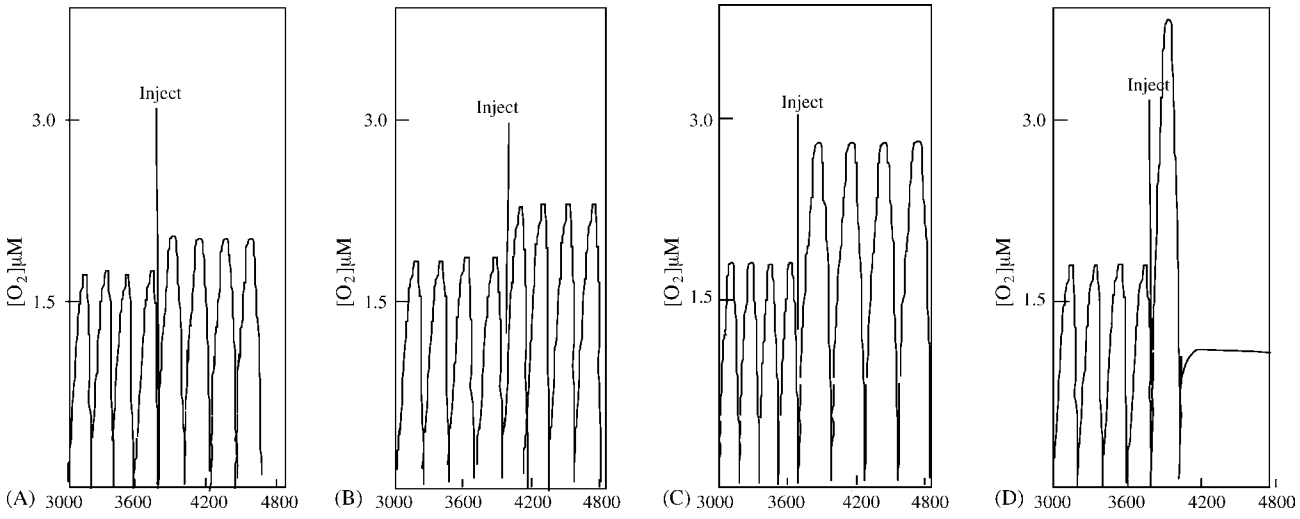

Fig. 3. Effect of peroxynitrite on simple periodic oscillation in the PO oscillation. The reaction mixture contained 6.0×10^{-8} mol/L MB, 3.5×10^{-5} mol/L DCP and 6.0×10^{-7} mol/L HRP, $T = 28(\pm 1)\,^{\circ}C$. The different concentrations of $ONOO^-$ from (A) to (D): (A) 2.5×10^{-8} mol/L, (B) 2.5×10^{-7} mol/L, (C) 7.80×10^{-7} mol/L and (D) 1.56×10^{-6} mol/L.

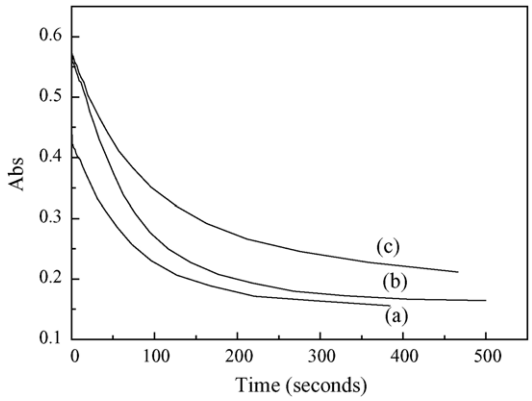

Fig. 4. Abs variation at 340 nm in PO oscillation perturbed by peroxynitrite of different concentration. Peroxynitrite concentration from (a) to (c): (a) 0, (b) 3.50×10^{-7} mol/L and (c) 3.5×10^{-5} mol/L. And the initial rate of NADH absorption changing at 340 nm (dA/dt) in 50 s from (a) to (c): (a) 0.00273 s^{-1}, (b) 0.00392 s^{-1} and (c) 0.00293 s^{-1}.

is Compound III [25] which can oxidize $NAD^{\bullet}$ into NAD^{+}. The absorption of Compound I is similar to that of native HRP. Compound II maximal absorbance is at 420 nm and Compound III at 418 nm. The absorbance detected at 420 was influenced by the two species [25]. The time-dependent spectra at 420 nm (Fig. 5) might be the co-effect of the two species. From Fig. 5 the kinetic curve at 420 nm changed significantly after added $ONOO^{-}$. The yielding intermediate increased more quickly which indicated peroxynitrite had effect on Compound III and Compound II. One of the two intermediates was influenced by peroxynitrite, the other would change according to the mechanism of the PO oscillation [12].

According to our previous experiments, peroxynitrite cannot oxidize NADH significantly whether catalyzed by HRP in the buffer. That showed the oxygen amplitude changing in the oscillation was not caused by the oxidation of NADH with peroxynitrite directly. Peroxynitrous acid (HOONO) readily reacts with various peroxidases, including HRP, to form the stable one-electron oxidation product Compound II [26]:

$$HRP^{3+} + HOONO \rightarrow \text{Compound II} + NO_2^{\bullet} + H^{+} \quad (2)$$

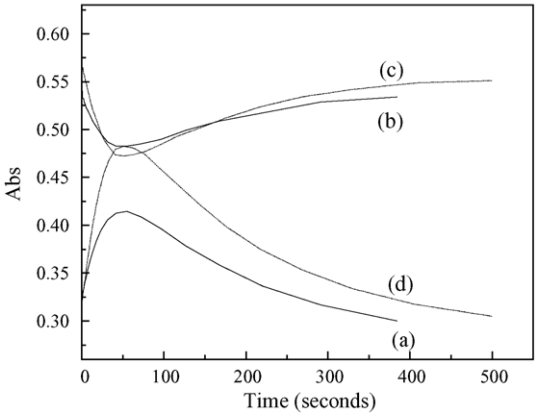

Fig. 5. Abs variation at 402 and 420 nm in PO oscillation with (dot line) or without (solid line) 3.50×10^{-7} mol/L peroxynitrite (a and d) at 420 nm, and (b and c) at 402 nm.

At pH 5.1 NaAc–HAc buffer, $ONOO^{-}$ could be converted into HOONO quickly, and reaction (2) could arise. NADH was a classical substrate, and Compound II can be reduced to native enzyme by NADH. In BSFO model for PO oscillation, Compound II can oxidize NADH as follows [12]:

$$\text{Compound II} + NADH \rightarrow NAD^{\bullet} + HRP^{3+} \quad (3)$$

But $ONOO^{-}$ could not significantly oxidized NADH directly catalyzed by HRP as mentioned above. It could be conferred that both Compound II and Compound III would be yielded. NADH could not reduce Compound III directly, while $NAD^{\bullet}$ can react with Compound III. The kinetic studies indicated trace $ONOO^{-}$ could accelerate NADH oxidization when peroxynitrite of low concentration was added into the PO system, and slow down NADH oxidization with high concentration peroxynitrite (Fig. 4). It could be concluded that trace enzyme intermediate Compound II would be yielded when low concentration peroxynitrite was introduced into the PO system, and the yielded Compound II would influence the oscillation but it would not destroy the oscillation. In the solution without PO system the yielded Compound II was too less to observe NADH oxidization. While NADH oxidization was accelerated through peroxynitrite transferring HRP to Compound II as function (2) and (3) in PO system which was very sensitive to the variety of the intermediates, and less dissolved oxygen was consumed in the reaction. The electrode would detect a higher amplitude of oxygen oscillation. This was consistent with the effect of $ONOO^{-}$ on the PO oscillation for experiment results (Fig. 3). When peroxynitrite of high concentration was introduced into the system, most peroxynitrite contributed to transfer HRP into Compound III and no enough NAD radical to react with Compound III to fit the oscillation cycle. Then NADH oxidation was slowed (Fig. 4c). That indicated HRP intermediates ratio in the oscillation system was destroyed which resulted the oscillation would be stopped as showed in Fig. 3D.

4. Conclusion

Peroxynitrite was found to perturbe the PO oscillation significantly and there was a linear relationship between the chang in the oscillation amplitude (I%) and the peroxynitrite concentration added in the range 2.50×10^{-8} to 1.56×10^{-6} mol/L. A highly sensitive method for the determination of peroxynitrite peroxynitrite was proposed based on this phenomenon. It was also found that peroxynitrite influenced PO oscillation system through the effect on HRP. The enzyme HRP intermediates in PO oscillation increased more quickly when peroxynitrite was introduced into the system.

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