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Bioelectrochemistry 63 (2004) 277–280
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Recombinant horseradish peroxidase- and cytochrome *c*-based two-electrode system for detection of superoxide radicals

Stepan Shipovskov[a], Elena E. Ferapontova[b,c,*], Irina Gazaryan[d,e], Tautgirdas Ruzgas[c]

[a] *Department of Molecular Biophysics, Lund University, P.O. Box 124, Lund SE 22100, Sweden*
[b] *Group of Bioinformatics, Web-lab, Novosibirsk IT Centre, Voskhod 26a, Novosibirsk 630102, Russia*
[c] *Department of Analytical Chemistry, Lund University, P.O. Box 124, Lund SE 22100, Sweden*
[d] *Department of Chemical Enzymology, Chemical Faculty, Moscow State University, Moscow 119899, Russia*
[e] *Burke Medical Research Institute, Mamaroneck Ave 785, White Plains, NY 10605, USA*

Received 23 June 2003; received in revised form 15 September 2003; accepted 19 September 2003

Abstract

The reliable detection of a superoxide anion radical $O_2^{\bullet -}$ is complicated by its spontaneous dismutation reaction to H_2O_2 at acidic pHs. To simultaneously detect both $O_2^{\bullet -}$ and H_2O_2 produced in the course of its spontaneous dismutation, an electrochemical two-electrode system based on cytochrome *c* (cyt *c*) and recombinant horseradish peroxidase (rHRP) was applied. Therewith, a limited applicability of the cyt *c* system for the reliable monitoring of $O_2^{\bullet -}$ in acidic and neutral solutions was shown. It was demonstrated that both the reaction of $O_2^{\bullet -}$ dismutation to H_2O_2 and the reaction between the formed H_2O_2 and $O_2^{\bullet -}$ chemically decrease the amount of the initially present $O_2^{\bullet -}$, decreasing the sensitivity and reliability of the electrochemical detection at acidic pH. However, by appropriately varying solution pH, the concentration of $O_2^{\bullet -}$ initially injected in the system can be estimated from the analysis of calibration curves for H_2O_2 obtained with highly sensitive rHRP-modified electrode system at pH 6.0 and 7.0.

Keywords: Superoxide radical; Hydrogen peroxide; Cytochrome *c*; Recombinant horseradish peroxidase; Bi-electrode system

1. Introduction

Free radicals, involved in the pathogenesis of cancer and some other human severe diseases, are commonly difficult to detect directly due to their high reactivity and thus a short life time period. It is known that they produce a cascade of damaging events in the living organisms leading finally to cell death [1–4]. The superoxide anion radical ($O_2^{\bullet -}$), a short-lived intermediate resulting from the $1e^-$ reduction of oxygen, is a primary component of so-called reactive oxygen species and is a potentially damaging agent, when its concentrations exceeds certain ‘‘antioxidant’’ level of the living system [1–3]. Development of the methods for the analysis of superoxide has gained considerable attention during the past decade [5–17]; reactions of superoxide with superoxide dismutase [5–8,14], superoxide reductase [9,10] and cytochrome *c* (cyt *c*) [11–17] were exploited for this purpose. Due to insufficient stability of the above-mentioned enzymatic systems, the most popular electrodes are based on the direct in vivo detection of $O_2^{\bullet -}$ with cyt *c*-modified electrodes [11–16]. Therewith, the reduction of cyt *c* by $O_2^{\bullet -}$ can be detected by means of a subsequent oxidation of reduced cyt *c* at the electrode surface:

$$O_2^{\bullet -} + \text{Cyt } c\ (Fe^{3+}) \rightarrow \text{Cyt } c\ (Fe^{2+}) + O_2 \xrightarrow{-1e^-} \text{Cyt } c\ (Fe^{3+}) \quad (1)$$

The re-oxidation current of cyt *c* is then proportional to the concentration of superoxide radical. However, the reliable determination of a real amount of $O_2^{\bullet -}$ present in the system is complicated by spontaneous dismutation of superoxide to H_2O_2:

$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (2)$$

In aqueous solutions the rate constant for reaction (2) increases more then 10^{12} times when changing from pH 14 to 7 [18]. Therefore it might be expected that under certain conditions the monitoring of $O_2^{\bullet -}$ can be accomplished through the analytical detection of hydrogen peroxide pro-

* Corresponding author. Tel.: +46-46-222-0103; fax: +46-46-222-4544.
E-mail addresses: elena.ferapontova@analykem.lu.se, ferapontova@nsk.fio.ru (E.E. Ferapontova).

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doi:10.1016/j.bioelechem.2003.09.026

duced in reaction (2). Previously, the possibility of the efficient direct (non-mediated) detection of H_2O_2 at gold electrodes modified with the recombinant forms of horseradish peroxidase (rHRP) was successfully demonstrated, at biologically important concentration levels of H_2O_2 (detection limit of 1 nM H_2O_2) [19–21]. In this work, a two-electrode system consisting of cyt *c* covalently bound to an alkanethiol-modified gold electrode [12–16] and a rHRP-modified gold electrode [19–21] was combined to follow transformations of $O_2^{\cdot -}$ in aqueous solutions at pH 6.0 (pH optimum for the analysis of H_2O_2) and pH 7.0 (physiological conditions).

2. Experimental

2.1. Chemicals

11-Mercapto-1-undecanoic acid (MU-COOH) and 11-mercapto-1-undecanol (MU-OH) from Aldrich; ethanol (99.6%) from Solveco Chemicals; cytochrome *c* from horse heart (cyt *c*), 1-ethyl-3(3-dimethyl) aminopropyl carbodiimide (EDC), potassium superoxide (KO_2), ethylenediaminetetraacetic acid (EDTA) from Sigma; hydrogen peroxide (H_2O_2, 30%) from Fluka; dimethylsulfoxide (DMSO) from Merck were used. Recombinant wild-type horseradish peroxidase (rHRP) produced in *E. coli* was from Dr. Irina Gazaryan [22]. All other chemicals were of analytical grade and were used as received. A 10 mM stock solution of KO_2 was prepared by adding DMSO (stored together with molecular sieve 4 Å) to KO_2 followed by 5 min sonication. Deionised Milli-Q water (Millipore, USA) was used in the work.

2.2. Instrumentation

Amperometry and cyclic voltammetry (CV) were performed with polycrystalline Au disk electrodes (CHI, 0.0314 cm^2) at (22 ± 1 °C) in a standard three-electrode cell containing two working electrodes (1 cm distance between the electrodes) and connected to bi-potentiostat AUTOLAB (PGSTAT 30, Eco Chemie). An Ag | AgCl | 0.1 M KCl and Pt wire were used as a reference and auxiliary electrodes, respectively.

2.3. Electrode modification

Immobilisation of rHRP at gold electrodes was performed as described elsewhere [17–19]. Detection of H_2O_2 with the rHRP-modified electrodes was done amperometrically at an applied potential of −50 mV. For modification of the electrodes with cyt *c*, the polished gold disk electrodes were electrochemically cleaned in 0.5 M H_2SO_4 by cycling the potential between −0.3 and 1.7 V. Then, the electrodes were washed with water and held in a 5 mM solution of MU-COOH and MU-OH in ethanol ([MU-COOH]/[MU-OH] ratio 1:3 [16]) overnight. After modification with the alkanethiol layer, the modified electrodes were placed in 30 μM solution of cyt *c* in 5 mM Na-phosphate buffer solution (PBS), pH 7.0, for 12 h. Then covalent binding of cyt *c* to MU-COOH/MU-OH-modified electrode was done by means of 5 mM EDC [16]. The quality of immobilisation was tested through the estimation of the peak areas of the cyt *c* oxidation–reduction processes from CVs of cyt *c*-modified electrodes (0.1 M Na-PBS, pH 7.0 and 6.0). The amperometric detection of $O_2^{\cdot -}$ was based on its oxidation at +0.13 V under constant stirring. $O_2^{\cdot -}$ was generated by injection of KO_2 in DMSO into the cell. The distance between the point of the KO_2 injection and the cyt *c* electrode surface was 1 cm and was kept constant during the experiment. The variations of that distance within 0.5 cm did not affect the results. The peak current was the calibration signal.

3. Results and discussion

The electrochemical two-electrode system was constructed to analyse simultaneously both superoxide and H_2O_2 produced in the course of a spontaneous dismutation of $O_2^{\cdot -}$. Fig. 1 shows the peak shaped current obtained with a cyt *c*-modified electrode and the steady-state current observed with an rHRP-modified electrode as a response to the addition of KO_2 into the electrochemical cell. The dependences of the electrochemical responses of the two electrodes on the concentration of added KO_2, at different pHs, are presented in Fig. 2a and b. As can be seen, the response of the cyt *c*-modified electrode to $O_2^{\cdot -}$ is linear within the studied concentration range, thus enabling its calibration with respect to [$O_2^{\cdot -}$]. In parallel, a linear concentration dependence of the electrochemical response of rHRP-electrode upon the injection of KO_2 was observed, resulting from the bioelectrocatalytic reduction of H_2O_2 produced in reaction (2). Therewith, due to high sensitivity of rHRP-electrodes to H_2O_2, the response of this electrode

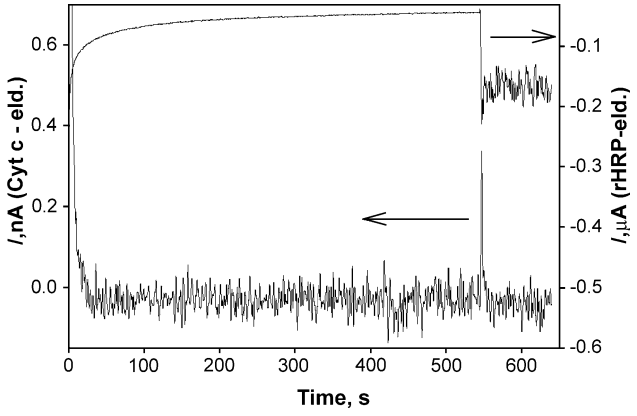

Fig. 1. Amperometric response of the rHRP- and cyt *c*-modified two-electrode system, at −0.05 and +0.13 V, respectively, upon the injection of KO_2 (the final concentration of 20 μM in the cell) in 0.1 M Na-PBS, pH 7.0.

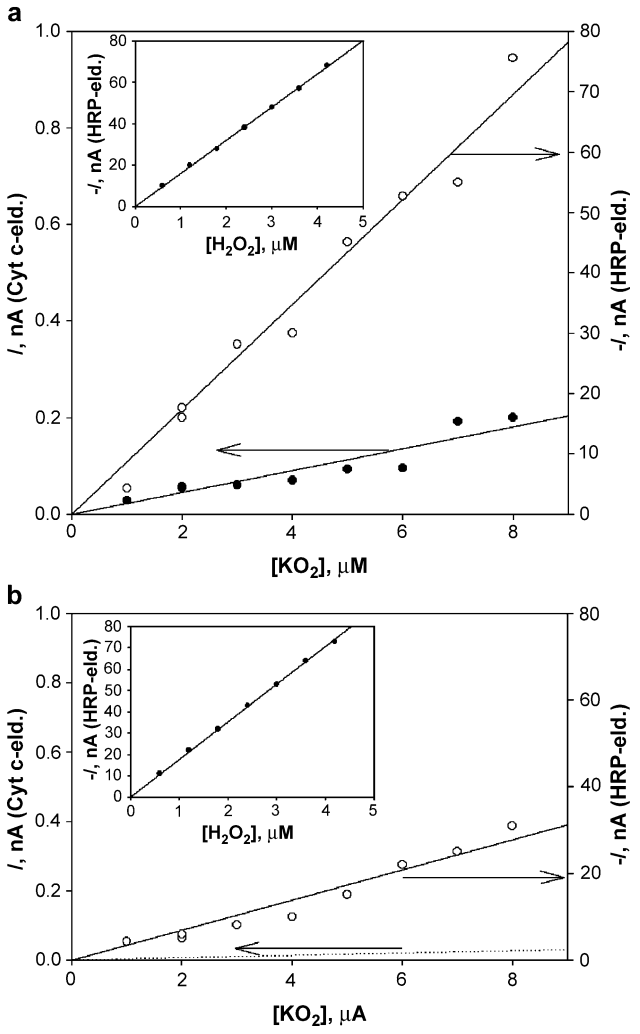

Fig. 2. (a) Calibration curves for superoxide obtained with cyt *c*- and rHRP-modified electrodes at applied potentials of +0.13 and −0.05 V, respectively, in 0.1 M Na-PBS, pH 7.0. Inset: calibration curve for H_2O_2 obtained with rHRP-modified electrode, conditions as in the main figure. (b) Calibration curves for superoxide obtained with cyt *c*- and rHRP-modified electrodes at applied potentials of +0.13 and −0.05 V, respectively, in 0.1 M Na-PBS, pH 6.0. Inset: calibration curve for H_2O_2 obtained with rHRP-modified electrode, conditions as in the main figure.

was sufficiently higher than that of cyt *c* (Fig. 2a). The detected signals from H_2O_2 produced during dismutation of $O_2^{\bullet -}$ were compared with the calibration curves obtained for H_2O_2 of known concentration (sensitivity 2 A M^{-1} cm^{-2} [21], Fig. 2a and b, insets). According to reaction (2), 2 mol of superoxide should produce 1 mol of H_2O_2. Keeping that in mind, the concentrations of H_2O_2 expected from 100% transformation of $O_2^{\bullet -}$ in reaction (2) were estimated and correlated with amperometric responses of the rHRP-modified electrode. However, when comparing the calibration curves obtained with the rHRP-modified electrode upon the addition of either KO_2 or H_2O_2, it became evident that the amperometric response to H_2O_2 produced in reaction (2) at pH 7.0 corresponds to about 70% of theoretically estimated $[H_2O_2]$.

Additionally, the response of the cyt *c*-modified electrode decreased drastically when changing from pH 7.0 to 6.0 (compare Fig. 2a and b), being virtually negligible at pH 6.0. Therewith, the efficiency of cyt *c* redox transformations at pH 6.0 and 7.0 is similar, and significantly less effective at more basic pHs (pH 8.0 and higher). Thus, the decrease in the response of the cyt *c*-modified electrode to superoxide additions evidently resulted from the higher instability of superoxide at lower pH [18], where instantaneous chemical transformation of superoxide should be favoured through reaction (2). However, differently from our expectations, no increase in the response (and consequently in the concentration of the detected H_2O_2) was obtained with the rHRP-modified electrode (Fig. 2b). On contrary, approximately a twofold decrease in the response of this electrode upon the addition of $O_2^{\bullet -}$ was observed when we changed from pH 7.0 to 6.0. The signal of the rHRP-electrode in this case corresponded just to 30% from the concentration expected theoretically according to reaction (2). Thus, other reactions involving $O_2^{\bullet -}$ and H_2O_2 have to be considered, which should depend on the solution pH. After analysis of the possible equilibria and the intermediate reactions in $O_2^{\bullet -}$ containing aqueous solutions [18], the following summarised reaction is suggested

$$2O_2^{\bullet -} + 2H_3O^+ + H_2O_2 \rightarrow 4H_2O + 2O_2 \quad (3)$$

As follows from reaction (3), in acidic solutions H_2O_2 produced in reaction (2) should immediately react with superoxide. This reaction contributes in the decreasing concentration of both H_2O_2 and $O_2^{\bullet -}$.

Thus, a limited applicability of cyt *c* system for the reliable detection of superoxide radical in acidic and neutral solutions is evident. Both the reaction of superoxide dismutation to peroxide (reaction (2)) and the reaction between the formed peroxide and superoxide chemically decrease the amount of the initially present $O_2^{\bullet -}$, thus decreasing the sensitivity and reliability of the electrochemical detection at acidic pHs.

Acknowledgements

Financial support from KVA, VINOVA, and the European Community (EC contract QLK3-CT-2001-00244) is greatly acknowledged. EEF thanks the Wenner-Gren Foundations for financial support.

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