Electrocatalytic Reduction of Nitric Oxide by Cytochrome P450-modified Gold Electrodes

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Received October 29, 2003

Key Words : Cytochrome P450, NO reduction, Modified electrodes, Impedance

Detection of NO has drawn much attention as NO plays important roles in physiological processes such as neurotransmission, muscle relaxation and immune system mediation.[1-3] Extensive work has been made to develop efficient electrochemical systems for the NO detection. Among them are metallo-porphyrins (MPs),[4] phthalocyanines (MPcs)[5] and metalloproteins such as hemoglobin[6] and myoglobin.[7] NO easily undergoes oxidation or reduction through the axial ligation to the active site.

Among heme-containing proteins, P450 class is of particular interest in that they are ubiquitous enzymes that are found both in prokaryotes and eukaryotes, catalyzing monooxygenation reactions for a great variety of organic compounds.[8,9] There have been efforts of utilizing P450s for the synthetic purpose in a regio- and stereo-specific manner. The natural use of P450 requires cofactors such as NAD(P)H and electron-transfer proteins such as P450-reductase, which is one of main obstacles to the commercial implementation. Estabrook[10] and Vilker[11] reported that it was possible to maintain catalytic activities of P450 for the substrate conversion when a cofactor was replaced by the electrode as an electron donor to P450 dissolved in solution. A redox mediator between the electrode and P450 may or may not be required depending on systems. As a nitric oxide reductase, cytochrome P450 accepts electrons from NAD(P)H. There are a number of works on the active site structure and NO reduction mechanism.[12-15] Here we report the use of cytochrome P450 adsorbed on the electrode surface as an electrocatalyst for the detection of NO through the direct electron transfer reaction.

Cytochrome P450 2B4 (CYP2B4) was purchased from Sigma and used as received. The P450 solution was made by dissolving 3 mg of CYP2B4 in 1 mL of tris buffer (10 mM) containing 10% glycerol. 10 μL was applied to the pre-cleaned gold surface (denoted as Au/P450) for 1 h and the electrode was washed with water before use. MPA (3-mercaptopropionic acid) treatment was done on the Au/P450 electrode. When treated with MPA, 5 μL of 10^{-3} M MPA solution was added to the Au/P450 electrode (denoted as Au/P450-MPA) and 30 min was allowed. Figure 1 shows the cyclic voltammograms of Au/P450 (curve a) and Au/P450-MPA (curve b) in pH 7.0 tris buffer solution. Without MPA, the voltammetry does not show any distinct redox peaks corresponding to the heme group. This is contrary to our expectation since unlike to other heme-containing proteins, the heme group in P450 is highly exposed so that the direct electron transfer can be possible, otherwise some molecular wirings may be needed. However, upon treatment with MPA, a distinct pair of redox peak was observed.

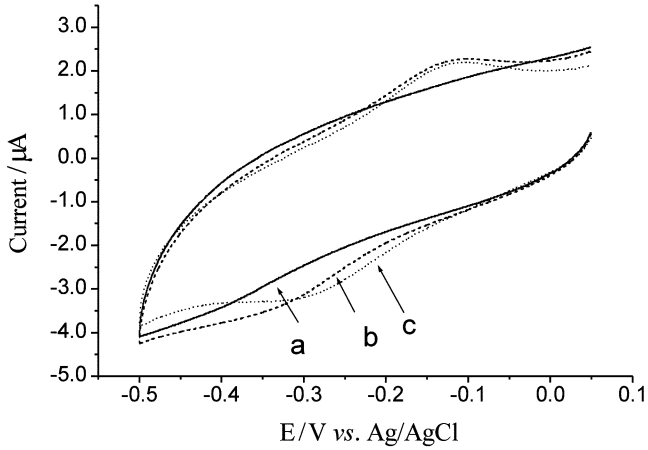

Figure 1. Cyclic voltammograms of adsorbed cytochrome P450 2B4 without (curve a, solid line) and with (curve b, dashed line) MPA. Curve c (dotted line) was obtained at a Au/P450-MPA electrode after injecting NO. Scan rate = 0.05 V s^{-1}; electrode area = 0.071 cm^2.

This is probably due to the fact that negatively charged MPA (pK_a = 6.0) provides a desirable environment for CYP2B4 (pI = 8.5) to undergo facile electron transfer reactions through electrostatic interaction between MPA and CYP2B4. This interaction can induce close packing of CYP2B4 on the surface and thus a favorable orientation toward electron transfer. The coverage from a voltammetric area was calculated to be *ca.* 9.0×10^{-11} mol·cm^{-2}, indicating a multilayer system, determined from the molecular size of 10 nm. The peak current was linearly proportional to the scan rate, showing that the enzyme was adsorbed on the surface. The peak separation slightly increased with scan rate having a formal potential ($E^{0'} = (E_{p,a} + E_{p,c})/2$) at *ca.* -0.22 V, suggesting a quasi-reversible electron transfer kinetics.

Impedance spectroscopic measurements with 2.5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (1 : 1), based on the Randles equivalent circuit[17] which describes the heterogeneous electron transfer reactions, also support that the observed electrochemical

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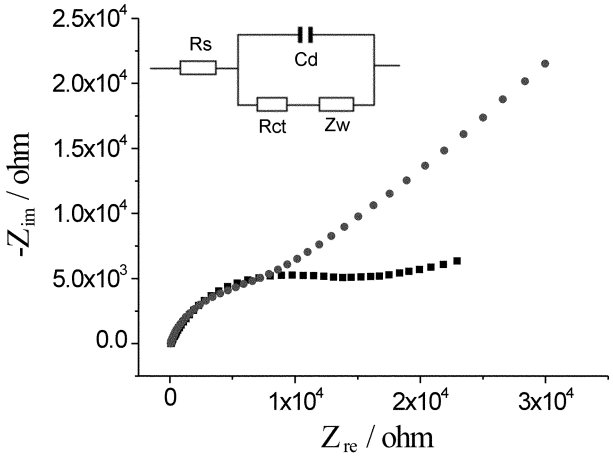

Figure 2. Nyquist plot of Au/P450 (■) and Au/P450-MPA (●) systems in the presence of 2.5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (1 : 1) couple. Potential was held at 0.22 V. Inset: Randles equivalent circuit to represent the system.

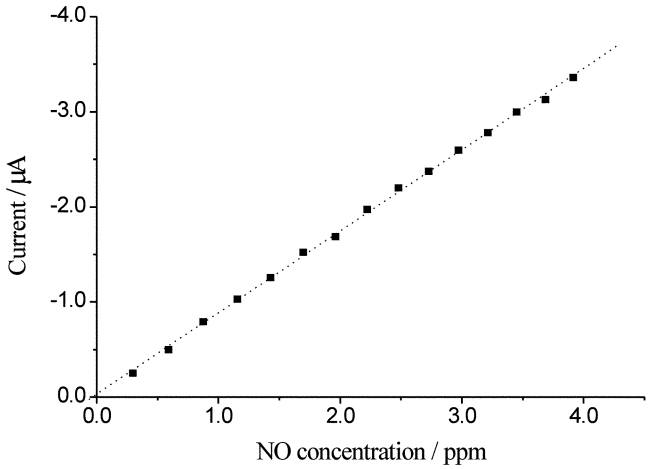

Figure 3. Plot of reduction current vs NO concentration from amperometric experiments. *r*-factor of fit = 0.9995; *y*-intercept = -0.032.

behavior comes from the MPA effect (Figure 2). The reduced charge transfer resistance of 8.6 kΩ for Au/P450-MPA from 14.8 kΩ for Au/P450 and the observed diffusional process of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ in the presence of MPA clearly show that MPA reorients CYP2B4 molecules in a way that fast electron transfer is possible. A linear part observed in the Nyquist plot indicates that $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox reaction is diffusion-controlled by exchanging electrons with CYP2B4.

Upon addition of NO, the reduction current was observed with a cathodic peak shifted by *ca*. 40 mV toward positive potential (Figure 1c). This suggests that NO binds to the ferric state of a heme group before reduction. The similar shift in the redox peaks of iron was observed when CO was added to the P450-adsorbed electrodes.[18,19] In that case, both cathodic and anodic peaks were shifted by more than 80 mV. However, neither CO reduction nor CO oxidation was observed, indicating CO simply binds to the iron site. A smaller potential shift in our case explains either NO does bind to Fe not as strongly as CO, or only some part of P450 is exposed to NO. Notable is that the anodic peak position did not change even with NO. This strongly suggests that once NO is reduced, the reduction product(s) does not remain on the surface. A catalytic reduction mechanism of in the case of formation of N_2O could be written as below.

$$(\text{heme})Fe^{3+} + NO \rightarrow (\text{heme})Fe^{3+}\text{-}NO$$
$$(\text{heme})Fe^{3+}\text{-}NO + H^+ + 2e^- \rightarrow (\text{heme})(Fe^{2+}\text{-}NO)^-\text{-}H^+$$
$$(\text{heme})(Fe^{2+}\text{-}NO)^-\text{-}H^+ + NO + H^+ \rightarrow (\text{heme})Fe^{3+} + N_2O + H_2O$$

The possibility of CYP2B4-modified electrode as a NO sensor was tested by amperometric measurements. A concentrated solution of nitrite was added to the iodide-containing solution at pH 2.0 while poising the potential at -0.37 V where the NO reduction took place. NO was produced by the catalytic decomposition of NO_2^-.[20]

$$2I^- + 2\,NO_2^- + 4H^+ \rightarrow I_2 + NO + 2H_2O$$

Figure 3 shows a linear response at least down to sub ppm concentration with an *r*-factor of 0.9995 and *y*-intercept of -0.0032 μA.

In conclusion, we demonstrated that cytochrome P450 could be electrochemically activated without a cofactor or a mediator. The direct electron transfer was possible between the electrode and CYP2B4 with an aid of MPA. CYP2B4-modified electrodes were used to detect dissolved NO.

Acknowledgment. This work was supported by Korea Research Foundation Grant (KRF-2002-070-C00066).

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