# Direct Electrochemistry of Nitrite Reductase from *Achromobacter cycloclastes* IAM 1013

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The cyclic voltammetry of the nitrite reductase from *Achromobacter cycloclastes* IAM 1013 exhibited well defined voltammetric response at a di-4-pyridyl disulfide (4-pyds) modified gold electrode in the presence of *A. cycloclastes* apopseudoazurin. The midpoint potential, $E_{1/2}$, of the native and type II copper-depleted (T2D) nitrite reductase obtained from the voltammogram were estimated to be 240 mV and 204 mV vs. NHE, respectively. The almost identical current value of the native and T2D reductase suggested that the voltammetric behavior contributed from the type 1 copper site. When nitrite was added into the nitrite reductase solution (pH 7.0), an enhanced sigmoidal cathodic current–potential curve indicating the catalytic regeneration of oxidized nitrite reductase was observed. The rate constant of nitrite reduction and the Michaelis constant, $K_m$, of the native reductase were estimated to be $5\times10^2$ $mol^{-1}\,dm^3\,s^{-1}$ and $7\times10^{-4}$ $mol\,dm^{-3}$, respectively. The rate constant of the nitrite reduction and the $K_m$ value of T2D nitrite reductase were also estimated to be $1\times10^2$ $mol^{-1}\,dm^3\,s^{-1}$ and $3\times10^{-3}$ $mol\,dm^{-3}$, respectively. These results suggested that the nitrite reduction activity is closely related with type II copper site.

Electron-transfer reaction is one of the most important reaction in biological systems. Many investigations of the biological redox systems involving electron-transfer chain coupled with ATP synthesis, biosynthesis of neurotransmitters, and metabolism of chemical materials have been performed.[1] Investigation of the direct electrochemistry for redox proteins at an electrode is very meaningful, for comprehending of the electron transfer, the driving force of the reaction, and the reaction mechanisms.[2] It is well-established that voltammetric studies offer not only redox potentials but also dynamic aspects such as kinetic analysis of electrode reactions of the several redox proteins and inter-protein electron-transfer reactions.[3] Direct electrochemical behavior of '*small*' electron-transfer proteins, cytochromes, blue copper proteins, and ferredoxins has been studied. These small electron-transfer proteins show the well defined electrochemical responses indicating fast electron-transfer at various promoter-modified gold electrodes, several metal oxide electrodes, and graphite electrodes interacting with functional groups of the protein.[4] Direct electrochemistry of '*large*' redox enzymes has been progressed in recent years, which is very prominent in order to understand the nature of the mechanisms of the enzymes. Biochemical implications of direct electrochemical response of several redox enzymes have been studied. The direct electrochemistry of methylamine dehydrogenase,[5] sulfide:cytochrome *c* oxidoreductase (flavocytochrome $c_{552}$),[6] and *p*-cresol methylhydroxylase[7] was observed at a modified gold electrode and a pyrolytic graphite electrode in the presence of polyamines as a promoter. The direct electrochemical response of the hydrogenase from *Alcaligenes eutrophus* Z-1 was reported.[8] It was accounted that the fast electron-transfer of cytochrome *c* peroxidase at graphite electrodes promoted by aminoglycosides[9] and the catalytic properties of the peroxidase at tin oxide electrodes were characterized.[10] Direct voltametric behavior of superoxide dismutase was measured at modified gold electrodes.[11] The fungal laccase, a multicopper protein, exhibited the direct electrochemical response at a graphite electrode in the presence of 2,9-dimethyl-1,10-phenanthroline.[12]

With these points in mind, we have studied the direct electrochemistry of a copper-containing nitrite reductase.

Nitrite reductases of denitrifying bacteria are divided into two types, heme-containing type and copper-containing type. Nine heme-containing nitrite reductases and eleven copper-containing nitrite reductases were isolated and characterized.[13] Copper-containing nitrite reductase (E.C. 1.7.99.3) of a denitrifying bacterium, *Achromobactor cycloclasters* IAM 1013 reduces nitrite ion to nitrogen monoxide as a part of the anaerobic respiration of nitrate to dinitrogen:[14]

$$NO_3^- \xrightarrow{2e} NO_2^- \xrightarrow{1e} NO \xrightarrow{1e} N_2O \xrightarrow{2e} N_2$$

It is well-known that the copper-containing nitrite reductase from *Achromobacter cycloclastes* IAM 1013 shows the intense absorption bands at 459 nm ($\varepsilon$ 2200 $mol^{-1}\,dm^{-3}\,cm^{-1}$), 582 nm ($\varepsilon$ 1800 $mol^{-1}\,dm^3\,cm^{-1}$), and 689 nm ($\varepsilon$ 1500 $mol^{-1}\,dm^{-3}\,cm^{-1}$) due to charge-transfer transitions in visible electronic absorption spectroscopy, and the EPR spectrum of the reductase indicates the existence of two types of copper, type I (blue) copper and type II (non-blue) copper in the protein molecule.[15]

Godden et al. reported the three-dimensional structure of *Achromobacter* nitrite reductase containing one type I copper and one type II copper per one subunit, and the enzyme exists as a trimer of the three iden-

tical 36 kDa subunits. Type I copper site is located 4 Å deep from the protein surface, two histidine, one methionine and one cysteine residues are coordinated to the type I copper center. The environment of the type I copper site is consistent with any other type I copper containing electron-transfer proteins. They also suggested that type II copper would be the substrate ($NO_2^-$) binding site located at the bottom of a 12 Å deep solvent channel.[16] It has been also proposed that the type II copper site is a $NO_2^-$ reduction site, and further reduction of NO produces $N_2O$ at the site by NO-rebound mechanism.[17] We reported the reversible disappearance of EPR signal of type I copper upon NO binding.[18]

Type I copper-containing pseudoazurin from *Alcaligenes faecalis* S-6 was found to be a specific electron donor to the nitrite reductase isolated from the same species.[19] Pseudozaurin from *Achromobacter cycloclastes* was also shown to be an efficient electron donor to the corresponding nitrite reductase.[20]

In this report we describe the cyclic voltammetric investigations on the direct electrochemical behavior and estimation of the electrochemical enzymatic reactivity of the nitrite reductase from *Achromobacter cycloclastes* IAM 1013.

## Experimental

**Materials.** Pseudoazurin and nitrite reductase from *Achromobacter cycloclastes* IAM 1013 were purified to $A_{280}/A_{594}$ ratio of 1.4 and $A_{280}/A_{456}$ ratio of 16.5, respectively, according to a slight modification of the previous procedures.[15] Apopseudoazurin was prepared by dialysis of a pseudoazurin-containing solution against 0.1 mol dm$^{-3}$ Tris-HCl buffer (pH 7.5) containing 10 mmol dm$^{-3}$ KCN for 48 h. T2D nitrite reductase was prepared by the previous method.[21] Protein homogeneity was checked with analytical polyacrylamide gel electrophoresis. Copper contents of nitrite reductase and pseudoazurin were determined by atomic absorption spectroscopy. The copper contents of native and T2D nitrite reductases were evaluated to be 4.5 and 3 per trimer of the protein, respectively. All protein-purification operations were carried out at 4 °C. All reagents used were of analytical grade or of the highest grade available, and distilled deionized water was used throughout.

**Spectroscopic Measurements.** Electronic absorption and circular dichroism (CD) spectra were measured with a Shimadzu UV-2200 spectrophotometer and a JASCO J-500 spectropolarimeter, respectively. Electron paramagnetic resonance (EPR) spectra (X-band) were recorded for the corresponding solutions at 77 K on a JEOL JES FE-1 EPR spectrometer. Copper content in the protein was determined by use of a Jarrell–Ash model AA-1 atomic absorption spectrophotometer.

**Electrochemical Measurements.** Cyclic voltammetry was carried out by using a BAS Model CV-27 Voltammograph (Bioanalytical Systems Inc.) with a Faraday cage and a Model PA-1 preamplifer.

Prior to each experiment, gold working electrodes were polished roughly with a 3-μm particle size of alumina-coated film (Sumitomo 3M), and again polished with a 0.3-μm particle size of alumina-coated film (Sumitomo 3M), sonicated for 1 min, rinsed thoroughly with distilled water. 4-Pyds modified gold electode (0.021 cm$^2$) was obtained by dipping the freshly polished gold electrode into a saturated solution of di-4-pyridyl disulfide.[22] Oxygen was removed from the working compartment by passing humidified oxygen-free argon through the cell for 15 min. A single-compartment electrochemical cell was used with an Ag/AgCl reference electrode purchased from Bioanalytical Systems and a platinum wire counter electrode separated by vicor glass tip from working solution, respectively. All potentials are referred to this reference electode (+205 mV vs. NHE at 25 °C).

## Results and Discussion

**Direct Electrochemistry of *Achromobacter* Nitrite Reductase.** It is generally difficult to observe the direct electrochemical behavior of relatively large molecular size of redox enzymes comparing with small electron-transfer proteins.[2] The molecular weight of nitrite reductase from *Achromobacter cycloclastes* is 108 kDa (trimer), and the observation of direct electrochemical response using normal techniques seems to be very difficult. In fact, no direct voltammetric response of nitrite reductase is observed at a 4-pyds modified gold electode (Fig. 1a). However, upon addition of apopseudoazurin[23] to the reductase solution, only one well-defined cyclic voltammetric response could be observed with a midpoint potential, $E_{1/2}$=240 mV (vs. NHE) and a peak separation, $\Delta E_p$=114 mV (Fig. 1b). The redox potential of nitrite reductase estimated from the cyclic voltammogram is consistent with the redox potential of copper-containing nitrite reductase from *Achromobacter xylosoxidans* NCIB 11015, which was found to be 260 mV vs. NHE.[25] Figure 2 shows the variation of peak current with the ratio of nitrite reductase (monomer=36 kDa) to apopseudoazurin (14 kDa)

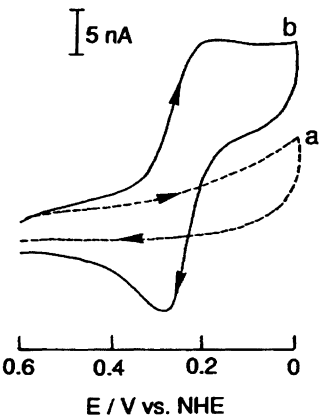


Fig. 1. Cyclic voltammograms of *A. cycloclastes* nitrite reductase in the absence (a) and the presence (b) of apopseudoazurin at a 4-pyds modified gold electrode at pH 7.0 (0.1 mol dm$^{-3}$ phosphate) at 25 °C. a: nitrite reductase (100 μmol dm$^{-3}$), b: apopseudoazurin and nitrite reductase (100 μmol dm$^{-3}$ each). Scan rate: 2 mV s$^{-1}$.

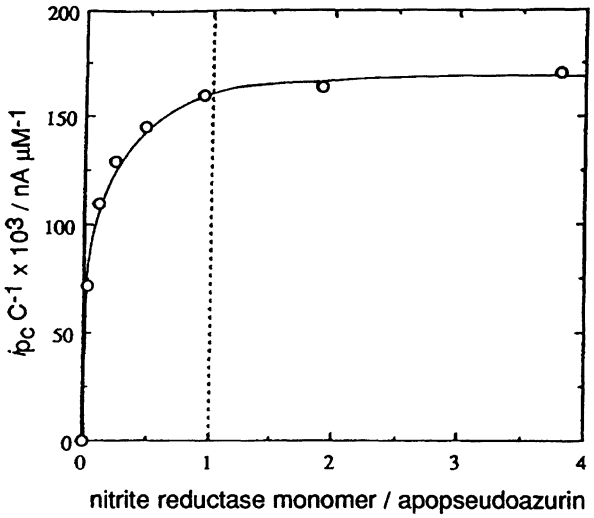


Fig. 2. The variation of relative peak current, $i_{pc}$ obtained from direct electrochemical response of nitrite reductase with molar ratio of nitrite reductase to apopseudoazurin at 25 °C. C is the concentration of nitrite reductase.

at a 4-pyds modified gold electrode.

No imporovement in current response (direct electrochemical response of nitrite reductase) corresponding to the promotion ability of apopseudoazurin was observed at the ratios of greater than 1, which can be explained by the 1:1 complex formation between the nitrite reductase and apopseudoazurin. Hill and co-workers reported the similar promotion of the electron-transfer of cytochrome $b_5$ by cytochrome $c$ at a pyrolytic graphite electrode surface which is unfavorable negatively charged for the direct electrochemistry of cytochrome $b_5$.[26] The complex formation between nitrite reductase and apopseudoazurin is also supported by the kinetic study of the oxidized reductase and the reduced pseudoazurin. Kashem et al. pointed out the possibility of the specific interaction between these proteins from the kinetic study.[20] Two-phases composed first-order and zero-order processes of reduction kinetics were observed for the 1:3 ratio of the nitrite reductase to pseudoazurin. However, when the molar ratio of nitrite reductase to reduced pseudoazurin was 1:1, only one kinetic process was observed. The X-ray crystallographic analysis of pseudoazurin from *Alcaligenes faecalis* S-6 indicated that the basic protein has positively charged patch constituted with basic amino acid residues near the copper coordination site located 6 Å deep from the protein surface.[27] The amino acid sequences of pseudoazurin from *Achromobacter cycloclastes, Methylobacterium extorquens* AM1, and *Alcaligenes faecalis* S-6 resemble each otehr.[28] The copper coordination sites and functionally important regions are well retained in each sequences. *Achromobacter* nitrite reductase is an acidic protein (isoelectric point, p$I$=4.1), and it may be possible to form the complex with positvely charged basic pseudoazurin through the electrostatic interactions of the protein surface. Thus the large enzyme, nitrite reductase might enable to access the electode surface and exchange an electron at the electrode.

Direct electrochemistry of T2D nitrite reductase also exhibited well-defined electrochemical response like that of the native reductase. The redox potentials of T2D nitrite reductase was estimated to be 204 mV vs. NHE ($\Delta E_p$=120 mV), which shifted to more negative potential than that of native enzyme. The negative redox-potential shift of T2D nitrite reductase might explain that the conformational change of type I copper environment is caused by the type II copper depletion, because the type I copper and type II copper sites are bridged by only two amino acid residues, His(135)–Cys(136).[29] The Cys and His residues are coordinated to the type I and type II copper sites, respectively, and the distance of the two copper sites is 12.5 Å (Fig. 3).[16] It has been suggested that the His(507)–Cys(508)–His(509) sequence in ascorbate oxidase is the electron-transfer pathway from type I copper site (close to the ascorbate binding site) to the trinuclear copper site (reaction site of oxygen reduction) via the His residues.[30,31] Additionally, Cys is coordinated to type I copper, and two His residues in that sequence are coordinated to binuclear type III copper center in ascorbate oxidase. In the case of the laccase which is believed to have the similar copper binding sites to those of ascorbate oxidase, the redox potential of the type I copper site in the native (429 mV) and T2D (430 mV) enzymes are essentially identical.[32] No structural change associated with the type II copper depletion was indicated from the redox properties and CD spectra.

**Estimation of Electrochemical Enzymatic Activity.** It is meaningful to estimate the enzymatic activities of redox enzymes by a direct electrochemical method. In generally, it is well known that the estimation of reductase-activity uses a large amount of reducing agent. If reductases are reduced directly at an electrode without denaturation, the direct electrochemical estimation of enzymatic activity should be able to avoid the using artificial reductant.

Addition of potassium nitrite to a 50 mmol dm$^{-3}$

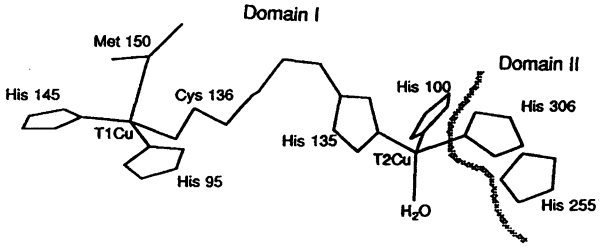


Fig. 3. Type I and type II sites of *A. cycloclastes* nitrite reductase taken from X-ray data by Godden et al. (Ref. 16). Domain I and II stand for different subunit in the trimer of nitrite reductase.

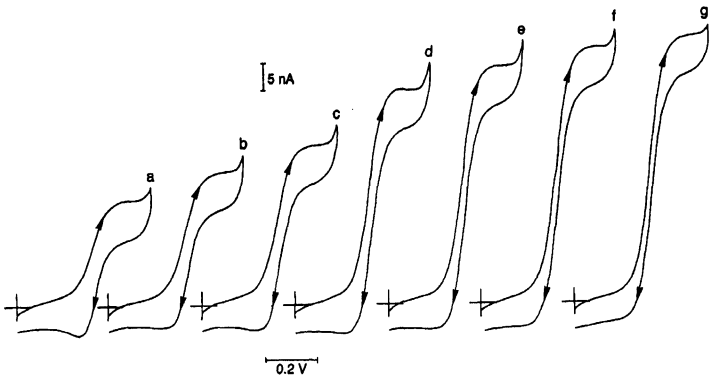


Fig. 4. The variation of catalytic responses for nitrite reductase (50 μmol dm$^{-3}$) in the presence of apopseudoazurin (100 μmol dm$^{-3}$) and nitrite, (a) 0.1, (b) 0.2, (c) 0.5, (d) 2.0, (e) 3.9, (f) 10.7, and (g) 17.4 mmol dm$^{-3}$ in 0.05 mol dm$^{-3}$ Tris–HCl buffer (pH 7.0) at 25 °C. Scan rate: 2 mV s$^{-1}$.

Tris–HCl buffer (pH 7.0) containing apopseudoazurin had no effect upon the appearance of the current–potential curve. This result immediately leads to the conclusion of no direct nitrite reduction ability of apopseudoazurin. When nitrite reductase was added into the apopseudoazurin solution with 1 : 1 molar ratio in the presence of excess nitrite, the shape of the voltammogram changed dramatically and an enchanced sigmoidal cathodic current-potential curve was observed (Fig. 4). Both the changes in the appearance of the voltammogram and the increased currents imply the catalytic regeneration of oxidized nitrite reductase in the diffusion layer, as shown in Fig. 5. The second-order rate constants of the nitrite reduction with native and T2D nitrite reductases were estimated to be $5\times10^2$ and $1\times10^2$ mol$^{-1}$ dm$^3$ s$^{-1}$, respectively, from the exiting theory for the catalytic process at an electrode.[33] We also obtained the activities of these reductases by a colorimetric method. The estimated enzymatic activities of native and T2D nitrite reductases are 370 and 190 μmol min$^{-1}$ mg$^{-1}$, respectively.[34] The unit of these activities refers to the consumption of nitrite by one mg of the reductase per minute. Both electrochemical and chemical assays displayed that type II copper depletion of nitrite reductase doses not inactivate the enzyme, but decrease the enzymatic activity of the native reductase.

Figure 6a presents the relative catalytic currents at various nitrite concentrations. The dependency of the catalytic current by electrogenerated reduced nitrite reductase on the substrate concentration corresponds to the general enzymatic kinetics, because we can predict that the catalytic current increases when the enzymatic activity becomes higher and higehr (Fig. 6b). The Michaelis constants, $K_m$, of native and T2D nitrite reductase were evaluated to be $7\times10^{-4}$ and $3\times10^{-3}$ mol dm$^{-3}$, respectively, from the linear plot of nitrite concentration against [nitrite concentration]/[observed catalytic current] following equation:

$$\frac{[NO_2^-]}{i_{cat}} = \frac{[NO_2^-]}{i_{max}} + \frac{K_m}{i_{max}}$$

where $i_{cat}$ is the observed catalytic current, $[NO_2^-]$ is the nitrite concentration, and $i_{max}$ is the maximum catalytic current. The former $K_m$ value is good agreement with the value of $5\times10^{-4}$ mol dm$^{-3}$ reported by Iwasaki et al.[15] These results suggest that the electrochemical estimation of redox enzymes is useful to evaluate the enzymatic activities.

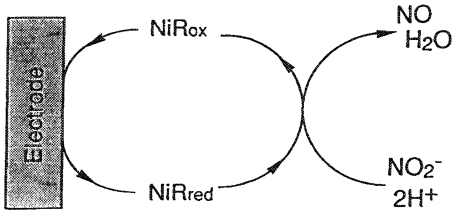


Fig. 5. Schematic representation of catalytic regeneration of enzymatically active reduced nitrite reductase at an electrode.

**Implications for the Type I and Type II Copper Sites.** It is very important to understand the functions of the type I and type II copper sites including the mechanisms of nitrite reduction. The estimated redox potential of the both native (240 mV vs. NHE) and T2D reductase (204 mV vs. NHE) are very resemble to those of other blue copper electron-transfer proteins, and hence the observed redox couple is assumed to be the type I copper site. The ratio of the cathodic peak currents of the T2D and native reductases show unity. This finding implies that the voltammetric response of the reductase is mainly contributed to the type I copper site which is the electron-transfer site at the electrode. It is well kbown that the type II copper site of the reductase is located 12 Å deep in a solvent channel formed by two monomers.[16] It might be impossible to observe the direct electron-transfer of type II copper site, because the type II copper site is far from the protein surface which interacts with electrode surface. The $K_m$ value for T2D nitrite reductase ($3\times10^{-3}$ mol dm$^{-3}$) suggests the less nitrite-binding ability compared with that of the native reductase ($7\times10^{-4}$ mol dm$^{-3}$). In addition,

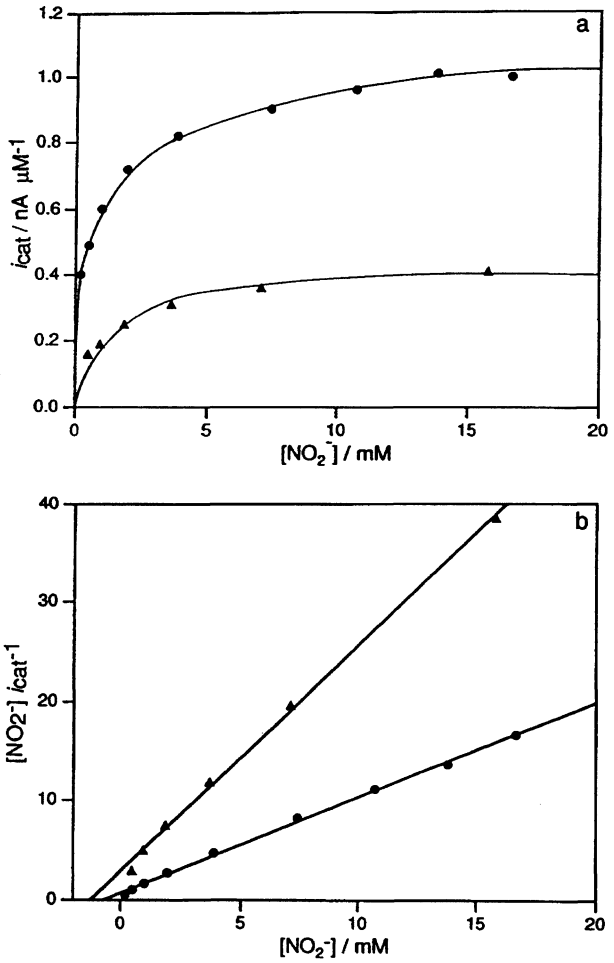


Fig. 6. (a) The viariation of catalytic currents with nitrite concentrations for native (●) and T2D (▲) nitrite reductases. (b) Linear plot of nitrite concentration with (nitrite concentration)/(observed catalytic current). Conditions: nitrite reductase, 50 μmol dm$^{-3}$; apopseudoazurin, 100 μmol dm$^{-3}$; 0.05 mol dm$^{-3}$ Tris–HCl buffer (pH 7.0); 25 °C.

the negative redox potential shift of type I copper by the type II copper depletion could affect decreasing the nitrite reduction activity. Therefore, it is assumed that the type I copper site is electron-transfer site to the type II copper site, and the type II copper site plays a role as nitrite binding and/or the reduction site.

The interfacial electron transfer of *Achromobacter cycloclastes* nitrite reductase was observed at a 4-pyds modified gold electrode in the presence of apopseudoazurin as a promoter. The observation of the direct electrochemical response of nitrite reductase implies the 1 : 1 complex formation between nitrite reductase and apopseudoazurin. These results evoke an electron-transfer aspect due to complex formation at the specific site via hydrogen bonding, salt bridge, and hydrophobic contact of *small electron-transfer proteins and large enzymes* as the native partner. The modified electrode surface is assumed to be electrostatically resemble to the cell membrane surface. Viewed in this light, the direct electrochemistry of the reductase promoted by apopseudoazurin can be regarded as a model of the redox reaction system at the periplasmic site.

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