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Selection of Mediators for Bioelectrochemical Nitrate Reduction

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Abstract The bioelectrochemical reduction of nitrate in the presence of various mediators including methyl viologen and azure A was studied using a 3-electrode voltammetric system. The catalytic potential for the reduction of the mediators was observed in the reactor, which for methyl viologen and azure A were -0.74 V and -0.32 V, respectively, with respect to the potential of Ag/AgCl reference electrode. This potential was then applied to a working electrode to reduce each mediator for enzymatic nitrate reduction. Nitrite, the product of the reaction, was measured to observe the enzymatic nitrate reduction in the reaction media. Methyl viologen was observed as the most efficient mediator among those tested, while azure A showed the highest electron efficiency at the intrinsic reduction potential when the mediated enzyme reactions were carried out with the freely solubilized mediator. The electron transfer of azure A with respect to time was due to the adhesion of azure A to the hydrophilic surface during the reduction. In addition, the use of the adsorbed mediator on conductive activated carbon was proposed to inhibit the change in the electron transfer rate during the reaction by maintaining a constant mediator concentration and active surface area of the electrode. Azure A showed better than nitrite formation than methyl viologen when used with activated carbon.

Keywords: nitrate reductase, azure A, bioelectrochemical reaction, voltammetry, mediator

INTRODUCTION

The electron-transfer cascades utilized by nature tend to be very high-yield, with little loss to unwanted reactions [1,2]. The key to this efficiency lies in an insulating protein shell and the ability of the charge-carriers to form highly selective complexes. However, the protein experiences different reaction conditions when separated from cell [3,4]. Electrons have to be transferred to the protein from other sources such as an electrode or a reducing agent like disodium dithionite [5,6]. Different kinds of electron mediator, therefore, are required to transfer electrons to the shielded active site of the protein [2,7].

Some molecular electron mediators have been used as electron carriers in artificial systems ranging from simple diffusion systems to complex immobilized systems [8,9], but most offer little selectivity in their electronic reactions as they can exchange electrons with any appropriate donor or acceptor that can approach them [5,10]. Therefore, selecting a mediator that has good selectivity in an enzyme reaction and good electron transfer efficiency is important in mediated enzyme reactions [8]. These are the key factors for the high efficiency and low power consumption during electron transfer.

Methyl viologen, benzyl viologen, organic dyes and other electrochemically active substances have been employed to mediate electron transfer [10,11]. These mediators were used for the electrical activation of soluble redox-enzymes lacking direct electrical contact with the conductive support. Cyclic voltammetry allows the effectiveness of a particular mediator to be assessed, using the theory for the catalytic electrochemical processes, and linear sweep voltammetry that of the electron transfer to the mediator to be determined under reaction conditions [12].

Soluble redox-enzymes electrically contacted by electron-transfer mediators with their own redox potentials provide different rate constants for the electron-transfer [13,14]. Comparison of the electron-transfer efficiency provided by different mediators in the presence of the same redox enzyme allows important parameters for the mediated electron transfer to be defined.

In linear sweep voltammetry, electrons are transferred to nitrate reductase, *via* the mediator from the electrode and then the reduced enzyme reduces nitrate to nitrite using the transferred electrons [8,13]. These consecutive reactions occur continuously in mixing reaction media. the direct determination of the nitrite produced in the

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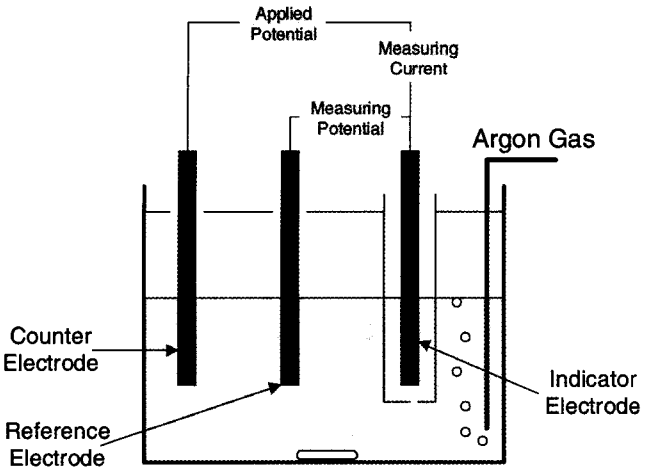

Fig. 1. Schematic diagram of enzyme reaction system with electrode. The three electrodes were dipped into the reaction media, which was homogeneously mixed using a magnetic stirrer.

electrochemical cell is very helpful in understanding the reaction cascade for each mediator in the electrochemical cell [8].

In this paper, the nitrite formation by a mediated enzyme reaction was observed for various mediators in an electrochemical cell and the usage of adsorbed mediator investigated to maintain constant mediator generation throughout the enzyme reaction.

MATERIALS AND METHODS

Electrochemical Cell

An electrochemical cell was especially designed for the enzyme reaction, which included mixing by a magnetic stirrer. The Electrochemical cell was maintained anaerobically by purging with argon gas, and the anode separated from the cathode and the reaction media by a sparger, as shown in Fig. 1. A commercial Ag/AgCl electrode was used as the reference electrode and graphite (diameter, 5 mm; length, 15 mm), from the Sigma-Aldrich Company (USA), as the material for the working and counter electrodes. Both the current and potential were measured at the working, reference and counter electrodes.

The potential of the working, electrode with respect to Ag/AgCl reference electrode, was determined based on the results of linear sweep voltammetry. The applied potential in the case of methyl viologen, for example, was -0.74 V, which was enough for the electrochemical reduction of methyl viologen. The potential of the working to the reference electrode was controlled by a voltammetry system.

Cyclic Voltammetry and Linear Sweep Voltammetry

Cyclic voltammetry and linear sweep voltammetry were carried out by applying a linear potential (that is, a potential that increases or decreases linearly over time) to the working electrode. As the potential is swept back and forth over the formal potential of the mediator in solution a current flows through the electrode that reduces the mediator. The magnitude of this current is proportional to the concentration of the mediator in solution. Both the current and potential were measured at the working, reference and counter electrodes.

Nitrate Reductase Assay

The assay mixture (total volume, 1.5 mL) was composed of 1 mM of each mediator as an artificial electron donor and 10 mM of the dithionite (reducing agent) in 80 mM potassium phosphate buffer (pH 7.0) solution. The addition of excess dithionite facilitated handling without an anaerobic chamber. The enzyme was added, and the mixture stirred at 30°C. Subsequently, the reaction was started by the addition of potassium nitrate (10 mM). Nitrate reduction was stopped by vigorously vortexing the mixture to oxidize all the dithionite and methyl viologen. After removing the cell extracts by centrifugation, the nitrite concentrations in the sample was measured.

Nitrite and Azure A Assays

To assay the nitrite concentration, 0.5 mL sulfanilamide solution (58 mM sulfanilamide in 3N HCl) and 0.5 mL NED solution (0.39 mM N-(1-napthyl) ethylenediamine hydrochloride in reagent grade water) were quickly added, with 1.5 mL reagent grade water added to 0.5 mL of the sample from the electrochemical cell, and then incubated at room temperature for 10 min. The concentrations of the nitrite and azure A were measured by their absorbance at 540 and 660 nm, respectively, using a spectrophotometer.

RESULTS AND DISCUSSION

Effect of Mediators on Enzymatic Nitrate Reduction

The tested compounds were well-known mediators and dye-stuffs [8,10,12,13] with molecular weights ranged from 257 (methyl viologen) to 997 (chicago sky blue). Five mediators including methyl viologen have their own reducible sites, so called electrophores, which can receive electrons directly from the electrode, and the four mediators are negatively charged to give channels for electron transfer. All of the charges of the tested mediators were stabilized by conjugated double bonds, such as phenyl and azo groups, and were soluble in water due to these charged functional groups, but some, such as benzyl viologen, became more insoluble when the salt form was reduced.

The relative activities of the tested mediators to methyl viologen, the most frequently used mediator in mediated enzyme reactions, are shown in Table 1. Nitrate ions were reduced to nitrite ions by the reduced form of the nitrate reductase, which had previously been reduced by the me-

Table 1. Relative activities of the various mediators compared to methyl viologen when tested under nitrate reductase assay condition

Mediator	Relative activity
Cationic mediators	
Methyl viologen (control)	1.0
Benzyl viologen	0.88
Azure A	0.31
Crystal violet	0.02
Safranin O	0.72
Anionic mediators	
Bromophenol blue	0.79
Cresol red	0.45
Cibacron blue	0.13
Chicago sky blue	0.01

diated electrons. The reducing condition was kept constant in the reaction media by addition 10 mM disodium dithionite to 80 mM potassium phosphate buffer, neutral pH.

Methyl viologen was found to be the best mediator among those tested when the formed nitrite concentration was compared using disodium dithionite. Benzyl viologen and safranin O also showed high activities for the mediated enzyme reaction as cationic mediators, but azure A showed relatively low and crystal violet showed almost no electron transfer activities.

Bromophenol blue had the best electron transfer activity in the anionic mediators, showing 80% of the activity of methyl viologen, whereas cresol red, cibacron blue and Chicago sky blue showed 45, 15, and 0% activities, respectively, with respect to that of methyl viologen.

Five of the cationic mediators are able to be reduced electrically because they have their own reducible sites, which are usually composed of negativity charged nitrogen or sulfur. Reduction of the mediators can be traced by cyclic voltammetry. As shown in Fig. 2, the cyclic voltammograms of each mediator show reduction and oxidation of the charged site at a certain potential to that of the reference electrode.

The potentials of the cyclic voltammogram tested ranged from 0.0 to 0.9 V relative to the potential of the Ag/AgCl reference electrode. Benzyl viologen was the only mediator that showed two reduction potentials in the given potential range, while the others showed only one. The reduction potential of azure A was the lowest, at 0.25 V, while the 2nd reduction potential of benzyl viologen was the highest, at 0.8 V, relative to the potential of the Ag/AgCl reference electrode.

The enzyme reaction usually occurs on mixing, which speeds the mass transfer in the electrochemical cell. However, voltammetry assumes no mixing: therefore, to simulate mediated electron transfer in an electrochemical cell, linear sweep voltammetry under mixing condition, was carried out for each mediator, as shown in Fig. 2.

When the applied potential was increased relative to

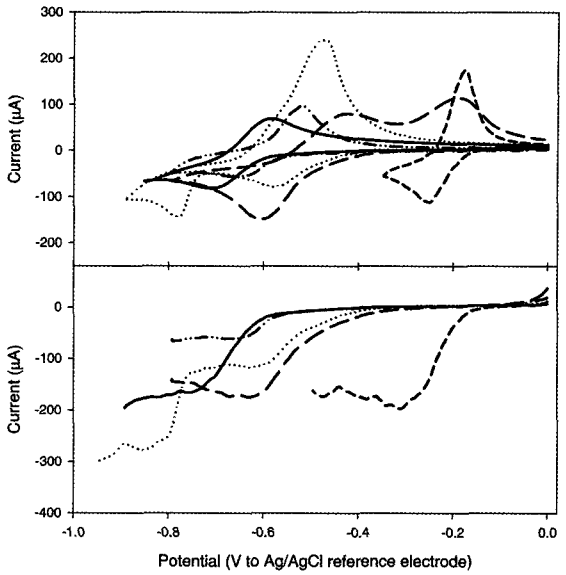

Fig. 2. Cyclic voltammograms and linear sweep voltammetry of tested mediators that could be electrically reduced in 30 mM phosphate buffer (—, 30 mM phosphate buffer; ····, methyl viologen; --, benzyl viologen; —··—, azure A; — —, crystal violet; —·—, safranin O).

Table 2. Potentials of the electrically reducible mediators tested

Mediators	Reduction potential (V) [a]
Azure A	- 0.32
Benzyl viologen (1st reduction)	- 0.59
Safranon O	- 0.61
Crystal violet	- 0.66
Methyl viologen	- 0.74
Benzyl viologen (2nd reduction)	- 0.83

[a] Potentials to Ag/AgCl reference electrode

that of the reference electrode, the current of the counter electrode increased abruptly at a certain potential, which remained constant with further increases in the potential. The reduction potential was read from linear sweep voltammetry signals. The 1st peak potential was regarded as the reduction potential of the tested mediator.

The reduction potentials of the 5 cationic mediators are listed in Table 2. Relative to the potential of the Ag/AgCl reference electrode, the reduction potential of azure A was -0.32 V, which was the highest potential among the mediators tested, and the 2nd reduction potential of benzyl viologen was -0.83 V, which was the lowest.

Nitrate was reduced enzymatically in the electrochemi cal cell for 60 min. The enzyme activity in the electrochemical cell was 0.07 unit/mL, with an initial concentration for each mediator of 1.0 mM. The potentials listed in Table 2 were applied to the electrochemical cell to effectively reduce the specificed mediator, with minimum side reactions. The nitrite formation for each me-

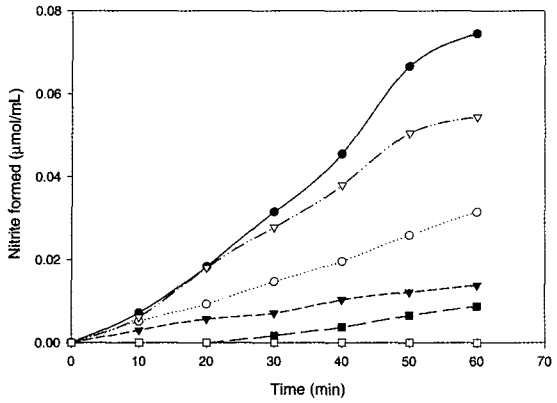

Fig. 3. Enzymatic reduction of nitrate to nitrite, using the various mediators that were able to be electrically reduced. The enzyme activity was 0.07 unit/mL, with a concentration of 1 mM for each mediator (●, methyl viologen; ○, benzyl viologen (at -0.59 V); ▼, benzyl viologen (at -0.83 V); ▽, azure A; ■, safranin O; □, crystal violet).

diator with respect to time was shown in Fig. 3.

Methyl viologen and azure A showed good electron transfer efficiencies in the mediated enzyme reaction, but benzyl viologen showed relatively poor activities under both the 1st and 2nd reduction conditions. This result came from the change in properties during the reduction. *i.e.*, reduced benzyl viologen tends to be adsorbed to the surface of working electrode due to the increase in hydrophobicity after the reduction. Thus, the active surface will be decreased after adsorption, and also the effective benzyl viologen concentration will be diminished in reaction media. Therefore, the mediated enzyme reaction under the 1st compared to the 2nd reduction conditions was better, even though more current was passed in the latter case.

The current of the counter electrode over a 60 min period are shown in Table 4. The highest passage of current and nitrite formation were observed when methyl viologen was used as the mediator, and the lowest current, but relatively high nitrite formation, was observed when benzyl viologen was reduced at -0.59 V.

Because the electrons for nitrate reduction come from the electrode, the nitrite formed should be proportional to the current at the counter electrode. To observe the current efficiency during a mediated enzyme reaction, the ratio of nitrite formed relative to the observed current was calculated from Figs. 3 and 4. Azure A showed the highest current efficiency, even though a relatively low current was observed. Benzyl viologen, under the 1st reduction potential, and methyl viologen also showed good efficiencies, while benzyl viologen, under the 2nd reduction potential, and safranin O showed low electron transfer efficiencies.

Pre-adsorbed Mediator to Inhibit the Adhesion of the Reduced Mediator

To compare methyl viologen and azure A during the

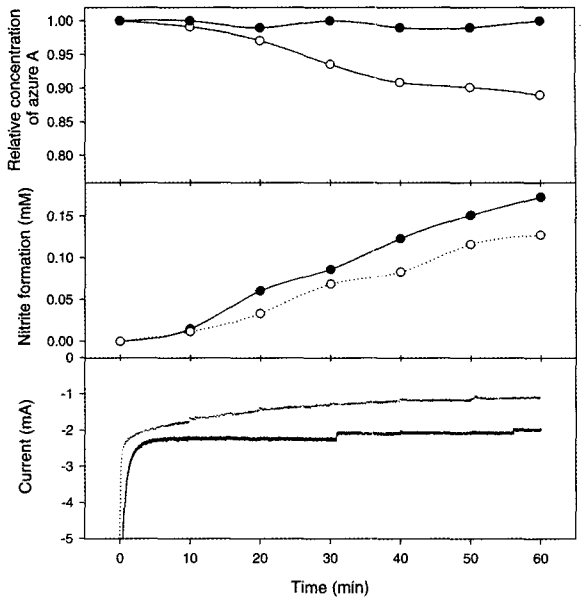

Fig. 4. Comparison between methyl viologen and azure A as a mediator for the enzymatic nitrate reduction. The residual concentration of azure A (the above), formation of nitrite (the middle) and current passed (the bottom), according to time, over one hour (●, —, methyl viologen; ○, ····, azure A).

mediated enzyme reaction, the nitrite formed and current passed were observed for 60 min, as shown in Fig. 4. The amounts of nitrite formed in each solution were similar for the first 10 min, but the difference between the two solutions increased thereafter. This phenomenon was also observed with the current at the counter electrode. The current passed decreased with respect to time when azure A was used as the mediator, but remained constant with methyl viologen.

To elucidate these effects in the electrochemical cell, the azure A concentration was measured with respect to time, and decreased by 10% after 60 min, indicating the attachment of 10% azure A to the working electrode surface. The decrease in the observed current was presumed to be due to the attachment of azure A to the working electrode from the measured concentration of azure A and observation of the electrode. A lower mediator concentration and decreased electrode surface area caused the low current passage through the electrode to be reduced.

The lack of a decrease in the current and concentration of methyl viologen were due to its electrophilic property, and even though one positively charged site was reduced by the electrode, the methyl viologen was still in soluble to buffer, solution and its concentration in the electrochemical cell remained constant.

Maintaining a constant mediator concentration, such as azure A, in the electrochemical cell is crucial for the bioelectrochemical nitrate reduction to proceed. Therefore, to maintain a constant current and inhibit surface electrode fouling, physically adsorbed azure A was used as the mediator in the electrochemical cell. The physical adsorption was achieved by the slow addition of activated

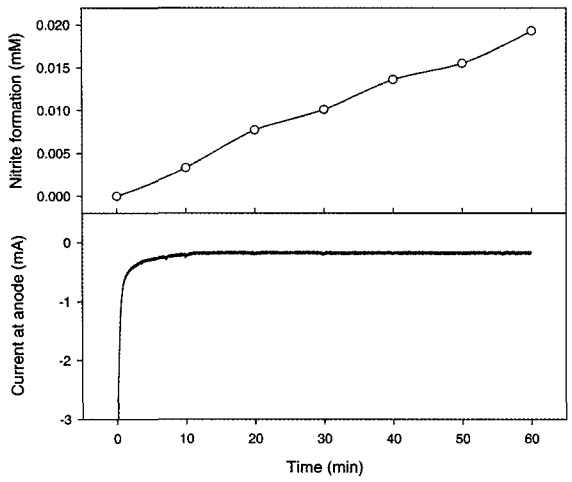

Fig. 5. Current passed and nitrite formation in the electrochemical cell using azure A physically adsorbed onto activated carbon. Almost the same current was passed through working electrode during the 60 min period.

Table 3. Formed nitrite concentration and currents at the various mediators during reduction

Mediators	Nitrite formed/ current passed (mM/mA mL)	Current (mA)
Azure A	0.23	0.25
Benzyl viologen (1st reduction)	0.22	0.14
Safranon O	0.03	0.36
Crystal violet	0.00	0.18
Methyl viologen	0.15	0.50
Benzyl viologen (2nd reduction)	0.04	0.31

carbon to the azure A solution at room temperature until the solubilized azure A disappeared, which means saturation of activated carbon at the activated carbon, and then washed with deionized water. Activated carbon was used as the supporting material due to its good electrical conductivity and high capacity for azure A adsorption. An electron could be transferred through the conductive activated carbon to azure A, with the reduced azure A mediating the enzymatic reduction of nitrate.

The azure A dispersed on the activated carbon at a molecular level could provide a favorable environment for the electrochemical reduction of a mediator and for the mediated enzyme reaction. Electrons were transferred from the activated carbon to the azure A, when they came into contact with the working electrode, due to the low resistance to electron transfer. Fig. 5 shows the current and nitrite formation in the electrochemical cell using azure A physically adsorbed onto activated carbon. The activated carbon saturated by azure A was used in electrochemical cell. An almost constant current and nitrite formation were observed through the counter electrode during a 60 min period, with no fouling by the azure A observed on the surface of the electrodes, but with the same electron efficiency shown in Table 3.

When an adsorbed mediator was used to inhibit the change in the electron transfer rate during the reaction, azure A showed better nitrite formation properties than methyl viologen.

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