Sensitized Fluorescence Generated in Bacterial Luciferase Catalytic Cycle with Metal Complex-binding Fluorescent Protein

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Summary: The $Ru(bpy)_3^{2+*}$-MV^{2+} sensitization system formed within a Nafion film was linked with *in vitro* bacterial luciferase reaction in the presence of a blue fluorescent protein. The electron released from the excited triplet $Ru(bpy)_3^{2+*}$ was transferred to the luciferase catalytic cycle with the fluorescent protein *via* electron relay, resulting in sensitization of the fluorescence protein.

Introduction

The bacterial luciferase reaction requires reduced riboflavin 5'-phosphate ($FMNH_2$), molecular oxygen and a long-chain aliphatic aldehyde to emit light peaking around 490 nm. In some bacteria, an endogenous accessory fluorescent protein acts as an *in vivo* emitter, leading to the change in color of bioluminescence due to the binding interaction between a luciferase intermediate and a fluorescent protein.[1] Another important feature of the bacterial bioluminescence is that luciferase links with the respiratory chain, *i.e.*, luciferase is probably fed with electrons from the respiratory electron flow.[2] From the viewpoints of the biological electron flow and of the biological energy transfer, it is of interest to characterize the relationship between the respiratory electron flow and the luciferase catalytic cycle with a fluorescent protein.

In this study, as a model for the electron rink between respiration and the bacterial bioluminescence, we attempted to couple a sensitization system, consisting of an excited tris(2,2'-bipyridinyl)ruthenium(II) ($Ru(bpy)_3^{2+}$) and N,N'-dimethyl-4,4'-bipyridinum cation (MV^{2+}), with the bacterial luciferase reaction in the presence of a fluorescent protein. As a trial basis, a Nafion film coated on electrode [3] was employed to provide a microstructure to accomplish

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the electron link of interest.

Materials and Methods

To prepare the Nafion film, the 0.5wt% Nafion ethanol solution was cast onto the electrode (25 μLcm^{-2}) and then allowed to evaporate slowly for 20 min. The cast - evaporation cycle was repeated trice. As conductive electrodes, optically transparent indium tin oxide (ITO) sputtered glass plate (9 × 40 mm) (ITO layer thickness = 1000 Å) and Au electrode (0.2 cm^2) were employed. To incorporate $Ru(bpy)_3^{2+}$, the Nafion coated electrode was soaked in an aqueous 2.5 mM $Ru(bpy)_3^{2+}$ solution for 15 min. The thickness of the Nafion film and the amount of $Ru(bpy)_3^{2+}$ incorporated were usually about 0.4 μm and about 50 nmol cm^{-2}, respectively. Subsequently, the $Ru(bpy)_3^{2+}$ incorporated Nafion film was equilibrated with the sample solution prepared in 0.1 M Na/K phosphate buffer (pH 7.0) for about 30 min. The Nafion film coated ITO electrode was used together with a gold 100-mesh gauze (8 × 25 mm) and a quartz plate (9 × 35 mm). The Au gauze was sandwiched between the Nafion film coated ITO electrode and the quartz plate. The assembled electrode was placed at right angle in a sample cup (400 μL). For the Nafion coated Au electrode, it was fitted up to face toward a photomultiplier tube. Potentials were quoted vs. a silver-quasi reference electrode, unless otherwise noted. Luciferase and blue fluorescent lumazine protein (referred to as BFP) used in this study were chromatographically isolated from the cells of *Photobacterium phosphoreum* according to the reported protocol. [4]

Results and Discussion

The cyclic voltammogram of the $Ru(bpy)_3^{2+}$ - Nafion film and corresponding emission profile are shown in Figure 1, together with the absorption spectral changes monitored during the potential scan. The absorption spectrum at 0 V was similar to that for $Ru(bpy)_3^{2+}$ in an aqueous solution. Upon scanning the potential positively at 2 mVs^{-1} from 0 V, the visible band with a maximum

around 450 nm decreased to the background level at about +1.2 V.

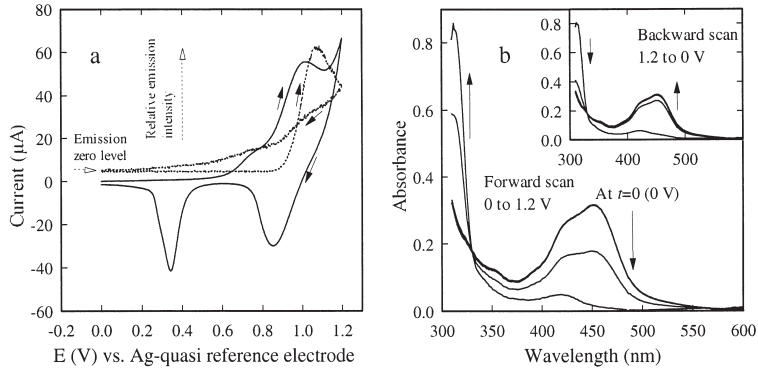

Figure 1. Cyclic voltammogram of the $Ru(bpy)_3^{2+}$ incorporated Nafion film equilibrated with 0.1 M phosphate buffer (pH 7) (solid curve) and corresponding emission profile (dotted curve) (a) and changes in absorption spectra monitored during the period of the potential scan (b). Scan rate = 2 mVs^{-1}. Each individual absorption spectrum was extracted at the 100-s intervals from the time-resolved absorption spectra.

To the contrary, a new band in the UV region markedly increased. The changes in absorption spectra were entirely restored during the backward scan in accordance with the redox behaviors of the $Ru(bpy)_3^{3+/2+}$ couple. It is noting that during the forward potential scan light emission is observed at potentials where $Ru(bpy)_3^{2+}$ is oxidized to $Ru(bpy)_3^{3+}$ (Figure 1a). The emission spectrum exhibited a maximum around 600 nm, suggesting that the $Ru(bpy)_3^{2+}$ d-π^* MLCT excited state[5] is produced within the Nafion film with no reducing agents added externally. There may be a possibility that the triplet excited $Ru(bpy)_3^{2+*}$ is produced with the aid of hydroxide ion as a reducing agent in the Nafion film.[6] The emission was not observed anymore when excess MV^{2+} was added to the system. This may be due to the oxidative quenching of $Ru(bpy)_3^{2+*}$ by MV^{2+}. When excess EDTA was further added to the system, no significant change in absorption band around 450 nm was observed. These observations may indicate that the $Ru(bpy)_3^{2+*}$ - MV^{2+} sensitization system is formed within the Nafion film coated on the electrode.

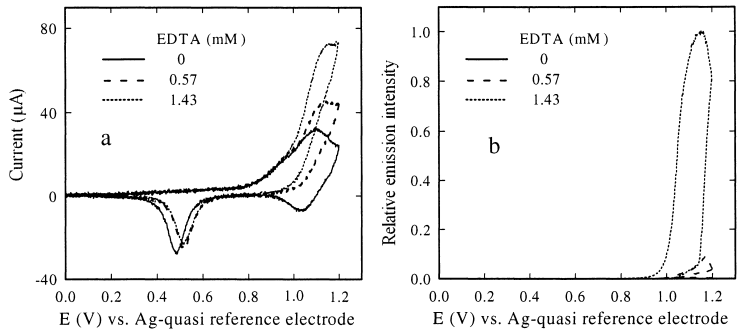

Figure 2. Cyclic voltammograms of the Nafion film equilibrated with luciferase reaction mixture (FMN = 0.5 mM, tetradecanal = 0.24 mM, luciferase = 6 μM) containing 1 mM MV^{2+} and EDTA in 0.1 M phosphate buffer (pH 7) (a) and the corresponding emission profiles (b). Scan rate = 20 mV/s.

Since the $Ru(bpy)_3^{2+*}$ - MV^{2+} sensitization system functions at the potentials much more negative than the reduction potential of FMN ($E^{\circ\prime}$ = - 0.22 V vs. NHE), it was predicted that the sensitizing system would be utilized as an electron source for the initiation of the bacterial luciferase catalytic cycle, requiring $FMNH_2$ in the first stage of the catalytic cycle.

To substantiate this prediction, light emission and redox behaviors were measured by using the $Ru(bpy)_3^{2+}$- Nafion film electrode under the various conditions. As a result, it was found that the electro-oxidation of $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$, followed by the formation of the excited $Ru(bpy)_3^{2+*}$, eventually induces light emission from the luciferase reaction mixture containing MV^{2+} and EDTA (Figure 2).

Based on the observation that the emission intensity increased with increasing EDTA concentration, it might be suggested that the electron released from $Ru(bpy)_3^{2+*}$ was transferred to the luciferase catalytic cycle *via* electron relay within the Nafion film. Unlike the isolated luciferase reaction (λ_{max}, ~ 490 nm), the emission maximum occurred around 510 nm. The observed red-shift was still present even when the FMN concentration was much lowered, suggesting that energy transfer interaction between a fluorescent luciferase intermediate and $Ru(bpy)_3^{2+}$ is present.

As stated above, one of noticeable features of the bacterial bioluminescence is that the endogenous fluorescent protein modulates bioluminescence. Taking this feature into account, the $Ru(bpy)_3^{2+*}$ sensitization system was coupled with the luciferase reaction in the presence of BFP. Figure 3 shows cyclic voltammograms of the Nafion film equilibrated with luciferase reaction mixture in the presence and absence of BFP and corresponding emission profiles. In the system with MV^{2+} and EDTA in large excess, it was found that the addition of BFP causes the increases not only in anodic current but also in emission intensity in the potential region where the $Ru(bpy)_3^{2+*}$ - MV^{2+} sensitization system links with luciferase. Noticeably, despite the presence of BFP, the emission maximum appeared at about 505 nm, which is located in longer wavelength region, compared with that for the normal luciferase reaction with BFP (λ_{max}, ~ 475 nm). As seen in Figure 3b, it is also notable that during the backward scan the emission decays rapidly in the system with BFP.

The results stated above may suggest i) that BFP stimulates the electron transfer from the excited $Ru(bpy)_3^{2+*}$ to luciferase *via* electron relay and ii) that an energy transfer interaction between the excited BFP* and the ground state $Ru(bpy)_3^{2+}$ exists. The observed faster emission decay may be an indication to support that the interaction between BFP* and $Ru(bpy)_3^{2+}$ is present within the Nafion film.

Conclusion

The $Ru(bpy)_3^{2+*}$ - MV^{2+} sensitization system formed within the Nafion film coated on electrodes was electronically linked with bacterial luciferase, leading to the initiation of the luciferase reaction. It is notable that the light emission occurs in the rather positive potential region where FMN is never reduced to $FMNH_2$ directly at the electrode surface.

In the luciferase reaction with BFP, it appears that the excited BFP* is efficiently produced in the luciferase catalytic cycle coupled with the sensitization system within the Nafion film and that the excited BFP* interacts with $Ru(bpy)_3^{2+}$, leading to a spectral red-shift.

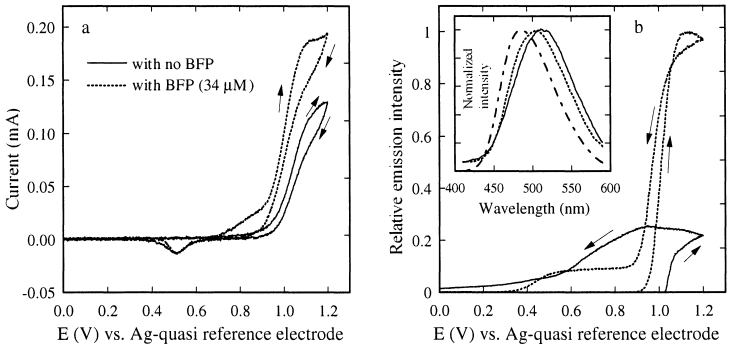

Figure 3. Cyclic voltammograms of the Nafion film equilibrated with luciferase reaction mixture (FMN = 50 µM, tetradecanal = 0.24 mM, luciferase = 7 µM, 5 mM = 1 mM and EDTA =5 mM) in 0.1 M phosphate buffer (pH 7) in the presence and absence of BFP (a) and the corresponding emission profiles (b). Scan rate = 20 mV/s. Inset, emission spectra recorded based on the potential step application; solid curve, with no BFP (0 to +1.3 V), dotted curve, with BFP (0 to +1.3 V) and chain and dotted curve, with BFP (0 to -1.1 V where FMN is directly reduced to $FMNH_2$ at the electrode surface).

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