Published in

Electrochemistry Communications 9 (2007) 2018-2022

An electroreflectance approach to study out the puzzling state of myoglobin
in a DDAB film on a pyrolytic graphite electrode surface

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Received 17 April 2007; received in revised form 16 May 2007; accepted 17 May 2007

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Abstract

The state of the heme of myoglobin molecules incorporated in a didodecyldimethylammonium bromide (DDAB) film on a pyrolytic graphite electrode was described using the results of electroreflectance measurements. It was found that the hemes are released from the myoglobin molecules. The ER spectrum of PG electrode | Mb-DDAB film was indistinguishable from the spectrum of PG electrode | hemin-DDAB film, even in the presence of NaBr, but clearly different from PG electrode | imidazole-coordinated-hemin-DDAB. These results support the claim of de Groot and coworker [M.T. de Groot, M. Merksx, M.T.M. Koper, J. Am. Chem. Soc. 127 (2005) 16224.; M.T. de Groot, M. Merksx, M.T.M. Koper, Electrochem. Commun. 8 (2006) 999.]. It is likely that DDAB is not a strong inhibitor of imidazole coordination but acts on the protein, resulting in conformational change and the heme release.

Keywords: Myoglobin; DDAB; Heme release; Electroreflectance spectroscopy; PG electrode

1. Introduction

Cationic surfactants or artificial lipids, represented by didodecyldimethylammonium bromide (DDAB), have been widely used as host film materials in protein electrochemistry after the report in 1993 from the group of Rusling [1]. They reported that myoglobin (Mb) molecules incorporated in a DDAB film are highly electroactive on a pyrolytic graphite (PG) electrode. The DDAB host film is believed to play roles: (1) to prevent impurities and denatured Mb from their direct adsorption on the electrode surface, which otherwise results in the formation of a blocking layer, (2) to *keep Mb molecules in a near-native conformation with intact embedding of the heme in the Mb-polypeptide backbone*, and (3) to highly facilitate the diffusion of Mb molecule in the film [2].

Recently, de Groot and his coworkers have claimed that “DDAB most likely induces the release of the heme group from the Mb” using voltammetric data, showing similarity between Mb-DDAB and hemin-DDAB films, and spectroscopic data of the Mb + DDAB dispersion [3]. In response to this, Guto and Rusling have published a paper with new data supporting their previous claim that Mb retains heme-embedding and near-native conformation in DDAB films [4]. A follow-up paper by de Groot and coworkers supporting the heme release has recently been published [5]. The state of heme in Mb-DDAB films is still in debate.

Because one of the authors of this paper (T.S.) was also one of the authors of a previous paper presupposing the near-native structure without heme release in artificial lipid films [6], we also should contribute to the solution of this issue. It is our understanding that the shortage of information about the state of the heme in the film on a PG electrode on the basis of an *in situ* spectroscopic approach has left this debate unsettled. We have a quite suitable tool, electroreflectance spectroscopy (ER), to analyze the state of electroactive chromophore even on a PG electrode with a low light reflectivity. Previously, one of the authors (T.S.) made preliminary ER measurements and was surprised with the similarity of the ER spectra between Mb-DDAB and

hemin-DDAB [7,8]. Recent debates inspired us to revisit ER measurements of Mb in DDAB more carefully and systematically.

With an aim at studying out the puzzling state of Mb in a DDAB film on a PG electrode surface, we herein describe the state of heme using the results of ER spectral measurements. Briefly, the ER signal is the ac component of reflectance of monochromatic UV-visible light at an electrode | solution interface under ac potential modulation while irradiating steady intensity light [9]. Although the surface of a PG electrode exhibits a low reflectivity, ER spectra have been successfully obtained so far for a number of dyes, including hemin, on the PG electrode surface [9-12].

2. Experimental

Myoglobin (horse skeletal muscle) from Sigma, hemin from TCI, and DDAB from TCI were used as received. 1-Octadecyl-1*H*-imidazole ($C_{18}Im$) was prepared in our laboratory. To prepare cast solutions of hemin and Mb, a 0.5 mM hemin (or Mb) in 0.01 M acetate buffer (pH 5.0) solution was mixed with an equal volume of a 0.01 M DDAB suspension in 0.1 M acetate buffer (pH 5.0). Before mixing, DDAB suspension was sonicated for a period of 3 h to be slightly white milky homogeneous state without precipitates. Before casting, thus prepared mixture was kept overnight at 4°C. These procedures are the same as those used by de Groot and coworkers [5]. A suspension of hemin (0.5 mM) + $C_{18}Im$ (10 mM) + DDAB (10 mM) in 0.1 M acetate buffer (pH 5) was prepared as a primary mixture sample. A piece of PG cut from the plate purchased from Tomoe Engineering, Tokyo, was connected to a lead with a silver paste and sheathed with an epoxy cement resin (Torr Seal, Varian) so that the basal plane is exposed as the electrode surface with its geometrical area of 0.25 cm². The electrode surface was polished with a 1500-grit emery paper and subsequently with a 0.05 μm Al₂O₃ slurry. After sonication in pure water, the surface was dried in air. The surface looked mirror-like so that the reflection image of the room lamp could be

seen without significant distortion. Then, 2.5 μL of hemin (or Mb)-DDAB cast solution was placed on the PG surface, and the electrode was dried for 30 min in air. All the measurements were conducted in an argon gas atmosphere and at room temperature ($22 \pm 2^\circ\text{C}$) using a quartz spectroelectrochemical cell. A Ag | AgCl | 3 M-NaCl reference electrode and a coiled Au wire counter electrode were used. The details of ER measurements are described in ref [9]. Non-polarized light with an incident angle of 32° with respect to the surface normal was used.

For the UV-visible absorption and circular dichroism (CD) spectroscopic measurements, respectively, Hitachi U-3000 and Jasco J-720 spectrophotometers were employed.

3. Results and Discussion

Cyclic voltammograms (CVs) for hemin-DDAB (a, dotted line) and Mb-DDAB (b, solid line) modified PG electrodes in 0.1 M acetate buffer solution (pH 5.0) at a potential sweep rate (ν) of 0.5 V s^{-1} are shown in Fig. 1.

Fig. 1 [See page 13](#)

The CV responses for both electrodes were in good agreement with the results of de Groot and coworkers [3,5], ensuring that we reproduced their experimental conditions. For both electrodes, the cathodic peak current (i_{pc}) was almost equal to the anodic one (i_{pa}), and both were proportional to ν in the range from 0.10 to 5.0 V s^{-1} , indicative of thin-layer electrochemistry. The dependence of the peak separation, the difference between the anodic peak potential (E_{pa}) and the cathodic one (E_{pc}), upon ν for the Mb-DDAB modified electrode was indistinguishable from that for the hemin-DDAB modified electrode. The formal potentials (E°), equated to the extrapolated value of $(E_{pc} + E_{pa})/2$ to $\nu = 0 \text{ V s}^{-1}$, were -0.200 V and -0.196 V , respectively, for hemin-DDAB

and Mb-DDAB modified electrodes. From the peak charges of CVs, *ca.* 21 % of hemin and 11 % of Mb relative to their cast amounts were found to be electroactive. The value for hemin exceeds the full monolayer adsorption amounts of hemin on a flat HOPG electrode [13].

Fig. 2 [See page 14](#)

Fig. 2 shows ER spectra for hemin-DDAB and Mb-DDAB modified PG electrodes. For Mb-DDAB (Fig. 2-b), all the cross points of real and imaginary part spectral curves are on the zero-ER line, not only at 14 Hz but also various other modulation frequencies (8, 44, and 88 Hz). This fact reveals that ER is looking solely at a single component redox response [9]. This indicates that, for the Mb-DDAB modified electrode, redox species detected by ER is either released hemin only or intact Mb molecules only, but not both, provided that intact Mb molecules, if being present, exhibit different ER spectral structures and electron transfer kinetics from those of released hemin. The relative magnitude ratio of imaginary part against real part is nearly the same for two different electrodes. This presumably indicates that the electron transfer kinetics and/or the product of interfacial capacitance and solution resistance are almost the same for the two electrodes [9]. The peak wavelengths are almost identical for these two spectra so that the differences between the two electrodes are less than 3 nm.

These facts reveal that the redox species and their oxidation-reduction processes in response to the potential modulation is the same for the two electrodes. The electroactive species giving rise to the ER signal in the Mb-DDAB film is not the native Mb but heme released from Mb molecules.

The situation in the Mb-DDAB film should be in between two extreme cases, namely:

- (1) At least 11 % of incorporated Mb molecules release hemes, which exclusively undergo redox reaction, while remaining intact Mb molecules with their bound hemes are electro-inactive or its electron transfer is very sluggish.

(2) All the Mb molecules release hemes, while due to the presence of remaining apo-Mb polypeptides, the CV response is smaller than hemin-DDAB film.

Fig. 3 [See page 15](#)

To specify when and how much the heme release took place, we measured UV-visible absorption and CD spectra for the solution before casting (Fig. 3). The Soret band absorption maximum of a clear Mb-DDAB solution at 398 nm was 8 nm shorter than that of Mb solution with a lower peak height. The Q-band peak positions of Mb-DDAB were different from those for Mb. For Mb-DDAB, no trace of peak or shoulder was observed at both 502 and 630 nm. In CD spectrum of Mb-DDAB, a negative-going peak at 220 nm was smaller than that at 206 nm, in sharp contrast to the spectrum of Mb solution. In addition, the positive-going CD spectrum of Mb solution in the Soret band region disappeared in the presence of DDAB (see inset).

These results reveal that the amount of Mb molecules with the intact heme is lower than detectable level. The near-complete heme release takes place before casting. This supports the case (2) above, unless drying process or potentiostatic condition results in quick back-incorporation of released hemes into apo-Mbs.

We should additionally notes the following aspects of the obtained ER spectra in Fig. 2. The spectral curves are quite similar to the curves for hemin directly adsorbed on graphite electrodes [11,14-16]. However, in light of the amount of electroactive species and the single-component signal feature, we look at hemin in the bulk film near the electrode but not only the adsorbed species. Because PG has large extinction coefficients in the UV-visible wavelength range, the ER spectrum is not necessarily in accord with the difference absorption spectrum [16]. In fact, both the ER spectra in Fig. 2 are different from the difference absorption spectra (absorption spectrum of reduced form from which that of oxidized form is subtracted) of hemin and Mb. Therefore, in order for final conclusion to be drawn, it should be experimentally ensured that the ER spectrum for an imidazole-coordinated hemin in a DDAB film distinctly

differs from the spectra in Fig. 2. Note that the 5th and 6th ligands of deoxy-Mb are an imidazole of histidine residue and a water interacting with an distal imidazole of histidine. If simulation of ER spectrum can be conducted easily without uncertainty, it would give the best supports of the state of heme. Because it is hardly made [16], we need experimental verification.

Dilution of primary hemin- $C_{18}Im$ -DDAB suspension by acetate buffer followed by centrifugation gave a clear supernatant of red color. Its absorption spectrum is shown in Fig. 4. Upon reduction, the Soret band peak shifted to a longer wavelength with absorbance enhancement, and sharp α - and β -bands appeared in the Q-band region at 559 and 529 nm, respectively. These absorption spectral features indicate that DDAB does not prevent $C_{18}Im$ from its coordination to iron center of hemin.

Fig. 4 [See page 16](#)

The primary suspension of hemin- $C_{18}Im$ -DDAB was diluted four times with 0.1 M acetate buffer solution to prepare the secondary sample in order for a clear solution to be used in the following experiments. The CV response of the PG electrode in this diluted hemin- $C_{18}Im$ -DDAB (line c in Fig. 1) exhibited redox peaks with $E^{0'}$ at 133 mV less negative potential than hemin-DDAB modified electrode at $\nu = 0.5 \text{ V s}^{-1}$. The peak current was proportional to ν , indicating that the deposit of hemin- $C_{18}Im$ -DDAB aggregates on the electrode surface underwent the thin-layer electrochemical reaction. The ER spectrum obtained in diluted hemin- $C_{18}Im$ -DDAB is shown in Fig. 5. For the real part spectrum, a very sharp negative-going peak at 421 nm and a positive-going horn at 439 nm were observed in the Soret band region. In addition, the double peak spectral structure of Q-band, corresponding to α - and β -peaks in absorption spectrum, was observed in 500-600 nm range. These characteristics of ER spectrum, being clearly different from those in Fig. 2 but rather similar to absorption spectra in Fig. 4, are of imidazole-coordinated hemin. The ER spectrum for a PG electrode, on which 2.5 μL of the secondary sample of hemin- $C_{18}Im$ -DDAB was cast and dried, also showed

almost the same spectral characteristics of imidazole-coordinated hemin (data not shown here).

Fig. 5 [See page 17](#)

These results tell us that, in both spontaneously formed deposition and cast film, C₁₈Im can coordinate to hemin, and that thus formed complex gives rise to the ER spectral feature with α - and β -peak characteristics. It is confirmed that ER spectral measurements are capable of distinguishing a hemin without coordinating imidazole ligands from a hemin with coordination. This, in turn, supports our interpretation of Fig. 2 that complete heme release takes place from Mb in a DDAB film.

We also found that the ER spectrum of hemin-C₁₈Im-DDAB cast film modified electrode is time dependent. The ER spectrum measured after a period of 20 min has passed after immersion of the electrode in acetate buffer solution still showed explicit features of imidazole coordination. After 24 h, however, the Q-band feature of the imidazole coordinated hemin was almost lost and the Soret band structure became similar to imidazole coordination-free hemin. This may indicate that, in the once dried cast film of DDAB, the imidazole coordination is not very stable in a long time range. In contrast when Mb is used, the ER spectral curve of Fig. 1-b was observed immediately after immersion. Therefore, it is most likely that dominant role played by DDAB to Mb is the introduction of the conformation change of Mb-polypeptide backbone that facilitates irreversible heme release. This interpretation is in line with the reports by Blauer and coworkers [17] and Tofani and coworkers [28] using other ionic surfactants than DDAB. It should be noted that the denature of an electron transfer protein in dialkyldimethylammonium bromide film is not always the case. To mention just one example, ferredoxin incorporated into a dioctadecyldimethylammonium bromide film on a PG electrode retains its native structure, giving rise to almost the same absorption spectrum of the [2Fe-2S] center as the native protein in solution phase [19,20].

It would be worthwhile to additionally list the results being important debate issues to be clarified.

- (1) Comparing the CD spectra in Fig. 3, we found that, irrespective of the presence of NaBr, the CD spectra for Mb with DDAB is different from that for Mb in the wavelength ranges of 190-250 nm and 350-440 nm.
- (2) The absorption spectra of Mb + DDAB solution and Mb + DDAB + NaBr solution (Fig. 3) exhibited an identical Soret band peak wavelength. But these spectra are far different from the spectrum of Mb solution in both Soret and Q-bands.
- (3) We measured CVs and ER spectra for a PG electrode modified with Mb (0.25 mM) + DDAB (5 mM) + NaBr (50 mM). The formal potential obtained from CV was approximately 30 mV less negative than that of (Mb + DDAB)-modified electrode. The ER spectral structure was almost the same as the curves in Fig. 2: two negative-going ER band peaks at 439 and 544 nm and two positive-going peaks at 476 and 608 nm were observed. The spectral curve shape was independent of the modulation frequency (8, 14, and 84 Hz).

These results clearly reveal that the catalytic activity of the PG surface [5,21] is not necessarily the cause of the heme release from Mb in a DDAB film. The heme release takes place before casting on a PG surface. Stabilization effect of the presence of NaBr on the intact heme structure of Mb was not reproduced in the present work. We are currently underway of the clarification of the reason of our observation of the absence of stabilization effect of NaBr in contrast to previous reports [1,2,4,5].

4. Conclusion

The results of our electroreflectance spectral measurements and other supporting experiments enabled us to conclude that heme of myoglobin is released in a DDAB cast film on a PG electrode.

Acknowledgements

This work is financially supported in part by a Grant-in-Aid for Scientific Research B (No. 16350077 for TS) from MEXT of Japan government. We thank Dr. M.T. de Groot for his kind answering our questions about experimental procedure details described in ref. [5] and Mr. Y. Narumi at Nagasaki University for his technical assistance in the synthesis of C₁₈Im.

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Figures

Figure 1 (revised) T. Sagara *et al.*,
An electroreflectance approach

Fig. 1. Cyclic voltammograms at $\nu = 0.50 \text{ V s}^{-1}$ in acetate buffer (pH 5.0) at a PG electrode ($A = 0.25 \text{ cm}^2$): (a) PG electrode|hemin-DDAB cast film, (b) PG electrode|Mb-DDAB cast film, and (c) bare PG electrode immersed in a hemin- C_{18}Im -DDAB suspension. The second cycles with an initial potential of 0.3 V are shown.

Figure 2 T. Sagara *et al.*,
An electroreflectance approach

Fig. 2. Electroreflectance spectra for (a) hemin-DDAB modified PG electrode and (b) Mb-DDAB modified PG electrode in acetate buffer (pH 5.0). The potential modulation is expressed as $E = E_{dc} + \Delta E_{ac} \exp(2j\pi f t)$, where the dc potential E_{dc} was set to be equal to E^0 , the amplitude of the modulation $\Delta E_{ac} = 141$ mV, $j = \sqrt{-1}$, and the modulation frequency $f = 14.0$ Hz. Both real part (solid line) and imaginary part (broken line) are shown. The former is the in-phase component of the ER signal with respect to E_{ac} and the latter is the 90°-out-of-phase component.

Figure 3 (new) T. Sagara *et al.*,
An electroreflectance approach

Fig. 3. UV-visible absorption (A) and CD (B) spectra for Mb (a, solid line), Mb + DDAB (b, dotted line), and Mb + DDAB + NaBr (c, dashed line). The absorption spectra (A) were measured by the use of a cuvette (1.0 cm light path length) with following compositions in acetate buffer (pH 5.0): (a) 0.025 mM Mb, (b) 0.025 mM Mb + 0.5 mM DDAB, and (c) 0.025 mM Mb + 0.5 mM DDAB + 5 mM NaBr. The CD spectra (B) were measured by the use of a cuvette (1 mm light path length). The concentration of Mb in acetate buffer (pH 5.0) for all the samples for (B) was 0.067 ± 0.017 mM with the same ratios of compositions of DDAB and NaBr as in (A). Note that a noise due to the optical filter exchange was observed around 390 nm in (A).

Figure 4 (renumbered) T. Sagara *et al.*,
An electroreflectance approach

Fig. 4. Absorption spectra of diluted hemin- $C_{18}Im$ -DDAB in acetate buffer. Solid line represents the spectrum of oxidized hemin and broken line does the spectrum of reduced hemin. Reduction was made by addition of an aliquot of sodium dithionite.

Figure 5 (renumbered) T. Sagara *et al.*,
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Fig. 5. ER spectrum of a bare PG electrode immersed in hemin- $C_{18}Im$ -DDAB. Both real part (solid line) and imaginary part (broken line) are shown. The ER measurement conditions were the same as those for Fig. 2.