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Protein Redox Potential Measurements Based on Kinetic Analysis with Mediated Continuous-Flow Column Electrolytic Spectroelectrochemical Technique. Application to TTQ-Containing Methylamine Dehydrogenase

Akihiro Sato, Masaki Torimura, Kazuyoshi Takagi, Kenji Kano,* and Tokuji Ikeda*

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502

Kinetic determination of protein redox potentials with a mediated continuous-flow column electrolytic spectroelectrochemical technique (CFCESET) is described. In this method, the redox state of the mediator is completely regulated by the continuous-flow column electrolysis, and the homogeneous redox reaction between the mediator and a protein sample in the column is monitored spectroscopically at the downstream of the column. The protein/mediator reaction is in the pseudo-first-order kinetics, and then the rate equation is analytically solved. The kinetic analysis provides the protein redox potential as well as the homogeneous rate constant. In the kinetic measurements, equilibration of the system within the column is not required, which allows the use of increased kinds of mediators. This method was successfully applied to quinoprotein methylamine dehydrogenase containing tryptophan tryptophylquinone (TTQ) as a prosthetic group. The kinetic aspect is also valuable for the thermodynamic analysis with the mediated CFCESET. The half-life time of the kinetics can be utilized to optimize the system for the attainment of the equilibrated state within the column and can provide the assurance that the system is in equilibrium.

The redox potential of biocomponents, especially of redox proteins, is one of the most important parameters characterizing bioenergetic systems. Development of a new method for convenient and reliable measurements of protein redox potentials continues to draw great attention. Direct cyclic voltammetry at conventional or modified electrodes is applicable only to limited species of relatively small metal proteins because of low heterogeneous electron-transfer rate constants of proteins (see refs 1−5 for reviews). Direct electrochemistry with a continuous-flow column electrolytic spectroelectrochemical technique (CFCESET) might be more useful, and successful results were obtained for various kinds of hemoproteins.[6−8] Most probably, surface conditions of the carbon fiber in the column would play an important role in the enhanced heterogeneous electron-transfer characteristics.

Indirect electrochemical techniques using suitable redox mediators, such as potentiometric-spectroscopic titration[9,10] or mediated optically transparent thin-layer spectroelectrochemical technique (OTTLSET),[11−13] are frequently employed for protein redox potential measurements. However, the methods require a long time for equilibration (in some case it takes 1 h for a given potential). In addition, time-dependent spectral change of mediators as well as protein after the potential step causes severe problems in the background subtraction, when spectral overlapping of protein and mediators occurs.

Recently, we have developed a mediated CFCESET for the thermodynamic determination of protein redox potentials.[14] This technique is based on the rapid redox regulation of a mediator solution by continuous-flow column electrolysis and an enhanced redox reaction between the mediator and protein sample injected in a flow-injection analysis (FIA) mode. The redox state of the equilibrated protein was monitored by a photodiode array spectroscopic detector. The background spectra of the mediator solution are time-independent and very stable due to the continuous flow, and thus, it is very easy to extract spectral information

* Corresponding authors. Fax: +81-75-753-6456. E-mail: (K. K.) kkano@kais.kyoto-u.ac.jp and (T. I.) tikeda@kais.kyoto-u.ac.jp.

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on the protein. The mediated CFCESET has great advantages over other indirect measurements because the experimental procedures and data treatments involve only simple handling and the measurements take little time.

The flow rate (f) and the column volume (V) determine the reaction time (t) in the mediated CFCESET. The t value is, however, limited in the range from ~20 s to 200 s in a conventional column electrolytic cell and HPLC pump apparatus. Therefore, it is essential, for the thermodynamic analysis, to select suitable mediators with homogeneous rate constants large enough to attain equilibrium during the indirect electrolysis in the column. It would also be important to establish a criterion for the attainment of the equilibrium, as in the case of other indirect electrochemical methods.

The rate equation of the homogeneous redox reaction in the mediated CFCESET can be analytically solved, because the concentrations of the oxidized and reduced forms of mediator remain constant in the column and then the homogeneous reaction between protein and mediator is in the pseudo-first-order kinetics.[14] This situation is another advantage of the mediated CFCESET and is in marked contrast with other indirect electrochemical methods.

In this paper, therefore, we establish kinetic methods for protein redox potential measurements on the basis of the mediated CFCESET. The kinetic approach will open a route for the mediated CFCESET to be extended as a more convenient and reliable method for the potential measurement. In this work, quinoprotein methylamine dehydrogenase (MADH) from *Paracoccus denitrificans* was used as a model redox protein. MADH has an $\alpha_2\beta_2$ subunit structure and contains one tryptophan tryptophylquinone (TTQ)[15] as a prosthetic group in each of the β subunits. The direct voltammetric signal of MADH was undetectable at conventional electrodes examined in our laboratory. The advantage and the kinetic aspects of the mediated CFCESET are discussed in detail.

EXPERIMENTAL SECTION

Reagents. MADH was purified from *Paracoccus denitrificans* as described in the literature.[16] Phenazine methosulfate (PMS^+) and phenazine ethosulfate (PES^+) were purchased from Wako (Kyoto, Japan) and Nacalai Tesque (Kyoto, Japan), respectively, and used without further purification. Phenazine methosulfate-2-sulfonate (PMS-S) was synthesized from PMS^+ as described in the literature.[17] All other chemicals were of analytical reagent grade and used as received.

Apparatus. The column electrolytic cell was received from Professor Kihara of the Kyoto Institute of Technology. The cell consists of a carbon wool working electrode packed tightly in a Bicole glass tube, an Ag/AgCl (saturated KCl) reference electrode, and a platinum coiled counter electrode.[18,19] All potentials were in reference to the Ag/AgCl electrode. The continuous-flow column electrolytic system used is illustrated in Figure 1. The system is almost the same as that described previously,[14] except that a precolumn electrolytic cell was attached on the upstream of the T-piece mixer to regulate the redox state of the mediator before the protein redox reaction. The electrode potential (E) of the main column and that of the precolumn were regulated so that they were identical with each other. The basal solution of the mobile phase was 0.1 M potassium phosphate buffer of pH 7.5 with an ionic strength of 0.5 M (with KCl). PMS^+, PES^+, and/or PMS-S were used as mediators, and the mediator solutions were prepared with the basal solution just before use. The mobile phase solutions in the reservoirs A and B were the basal solution and a mediator solution usually at 50 μM, respectively, and the temperature was controlled at 25.0 ± 0.1 °C in a water bath. The two solutions were given identical flow rates through the use of HPLC pumps. The mobile phase and the outer electrolytic solution (sat. KCl) of the column electrolytic cells were continuously bubbled with argon gas during the experiments. Stainless steel tubing was used for the electrolytic solution line to prevent the dioxygen contamination. All experiments were performed at a room temperature controlled at 25 ± 3 °C. All other details of the apparatus used were described in a previous paper.[14]

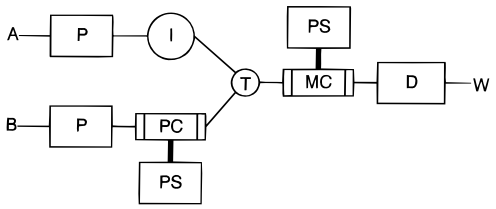

Figure 1. Schematic diagram of a mediated CFCESET system. A, reservoir of the basal solution (0.1 M potassium phosphate buffer of pH 7.5 with an ionic strength of 0.5 M with KCl); B, reservoir of a mediator solution; P, HPLC pump; I, injector; T, T-piece mixer; PC, precolumn electrode for the regulation of the mediator solution; MC, main column electrode for the indirect redox reaction of protein; PS, potentiostat; D, photodiode array detector; W, waste.

Procedures. After stabilizing the background spectrum of the electrolyzed mediator solution at a given E and at a given flow rate (f) (usually 10 min after each potential step), a 10-μL portion of an MADH solution was injected on the mobile phase of reservoir A in the FIA mode. The effective volume of the main column (V) was estimated as 1.20 mL from the difference in the retention time of the FIA peak with and without the main column. The inner volume of the line from the T-piece mixer and the top of the main column and from the end of the main column to the detector (0.05 mL) was negligibly small compared with V. Thus, we estimated the time of the redox reaction between protein and mediator (t) by $t = V/f$. All experiments were performed at room temperature. Other details are described in a previous paper.[14]

RESULTS AND DISCUSSION

Redox Behavior of Mediators. Figure 2A shows the background spectra of the electrolysis solution containing 50 μM PMS^+ as a mediator at various values of E. The appearance of clear isosbestic points at 276 and 349 nm indicates a one-step two-electron-transfer mechanism without generation of a detectable amount of the intermediate radical. The E dependence of the

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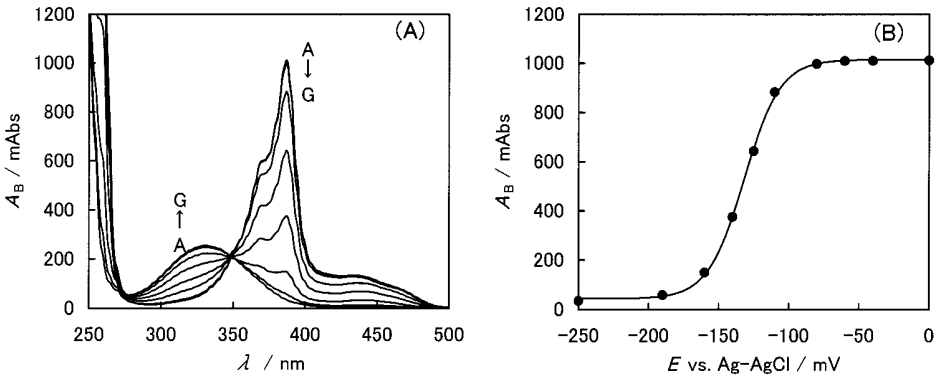

Figure 2. Panel A: Background absorption spectra of the PMS^+ solution (50 μM) regulated by the CFCESET at $f = 2.0$ mL min^{-1}. $E =$ (A) −0.080 V, (B) −0.110 V, (C) −0.125 V, (D) −0.140 V, (E) −0.160 V, (F) −0.190 V, (G) −0.240 V. Panel B: Background absorbance (A_B) at 387 nm as a function of the electrode potential (E). The solid line represents the regression curve obtained by nonlinear analysis on the basis of eq 1 with $E^{o\prime}_M = -0.132$ V.

background absorbance (A_B) at 387 nm satisfied the following Nernstian equation as shown in Figure 2B.

$$A_B = (\epsilon_{M,ox}[M_{ox}] + \epsilon_{M,red}[M_{red}])l = \frac{(\epsilon_{M,ox}\eta_M + \epsilon_{M,red})[M]_o l}{\eta_M + 1} \quad (1)$$

with

$$\eta_M \equiv \frac{[M_{ox}]_{eq}}{[M_{red}]_{eq}} = \exp\left[\frac{2F}{RT}(E - E^{o\prime}_M)\right] \quad (1a)$$

where $[M_{ox}]$ and $[M_{red}]$ are the concentrations of the oxidized and reduced forms of the mediator, respectively, while $[M]_o$ is the total concentration of the mediator ($= [M_{ox}] + [M_{red}]$) and the subscript eq denotes the equilibrated state. $\epsilon_{M,ox}$ and $\epsilon_{M,red}$ are the absorption coefficients of M_{ox} and M_{red}, respectively, and l is the light-path length. $E^{o\prime}_M$ is the formal redox potential of the mediator. Nonlinear regression analysis of the A_B at 387 nm vs E plots on the basis of eq 1 yielded -0.1317 ± 0.0005 V as $E^{o\prime}_M$ of PMS^+. In the analysis, $\epsilon_{M,ox}[M]_o l$ and $\epsilon_{M,red}[M]_o l$ were evaluated experimentally from the absorbance at $E \gg E^{o\prime}_M$ and $E \ll E^{o\prime}_M$, respectively. Similar background absorption spectra were obtained for PES^+ and PMS-S. The $E^{o\prime}_M$ values were evaluated as -0.155 ± 0.003 V for PES^+ and -0.048 ± 0.004 V for PMS-S. The evaluated $E^{o\prime}_M$ values of PMS^+ and PES^+ are very close to those in the literature[20] (PMS^+, −0.132 V; PES^+, −0.157 V at pH 7.5), while a slight difference was observed for PMS-S from the reported one (−0.06 V at pH 7.5 by the polarographic method[21]). All these results support the idea that the mediators used here are totally reversible in the electrode process and exist as the equilibrated state in the electrolytic column. Since the mediator concentration is low enough (50 μM in this work), no detectable effect of uncompensated ohmic drop was observed in electrolysis solutions with the ionic strength down to at least 0.1 M.

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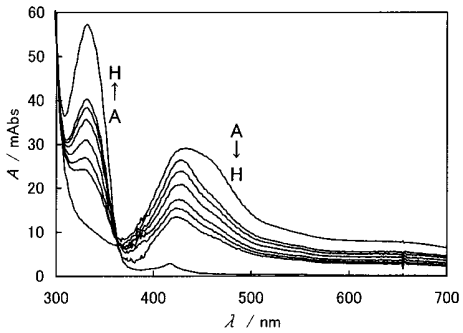

Figure 3. Background-corrected absorption spectra of the FIA peak of MADH (50 μM × 10 μL) in the presence of 50 μM PMS^+ at $E =$ −0.080 V. The reaction time t ($= V/f$) is (B) 18 s, (C) 24 s, (D) 36 s, (E) 48 s, (F) 60 s, (G) 72 s. The spectra (A) and (H) correspond to the fully oxidized and reduced MADH, respectively, and were obtained at $E = -0.000$ V and −0.250 V, respectively, at $f = 60$ s. The absorption spectra were corrected for the f dependence of the dilution factor, which was evaluated from the MADH peak height at $E = 0$ V.

Kinetic Analysis of the Redox Reaction between MADH and Mediators. When a 10-μL portion of an MADH solution (50 μM) was introduced into the column in the mode of FIA, the shape and the retention time of the MADH peak were practically independent of E, suggesting no adsorption of MADH on the column electrode surface. Judging from the peak height, the injected MADH solution was diluted about 50−70 fold at the peak top (0.7−1 μM), depending on f. The concentration ratio of PMS^+ and MADH was then about 50−70 at the peak top. The dilution factor was practically independent of E.

The absorption spectra of MADH and PMS^+ are overlapped with each other. Because the background spectra of PMS^+ was stable and constant in this method, through background subtraction it was very easy to extract the spectral information of MADH, even though the absorbance of MADH was very small compared with that of PMS^+. The absorption spectra of MADH were dependent on t (which was controlled by f), as shown in Figure 3. Similar t dependence of the MADH spectra was observed at the other E values. The result means that the reaction between MADH and PMS^+ did not reach the equilibrated state in the column under the present conditions.

During the spectral change, an isosbestic point appeared at 363 nm and there was no spectral evidence for the generation of the TTQ semiquinone intermediate as reported in the literature.[22] These results support an (apparent) one-step two-electron transfer in the mechanism for the MADH redox reaction. Therefore, the reaction between MADH (P) and PMS^+ (M) can be written as follows:

$$P_{ox} + M_{red} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} P_{red} + M_{ox} \qquad (2)$$

The reaction between protein and mediator would proceed according to Michaelis-Menten kinetics. When the mediator concentration is sufficiently lower than the Michaelis constant (K_M), however, the reaction becomes a simple second order in the kinetics and the rate constants k_f and k_b can be expressed by k_{cat}/K_M for the forward and backward reactions, respectively, where k_{cat} is the catalytic constant. In our experiments, $[M_{ox}]$ as well as $[M_{red}]$ is equal to or lower than 50 μM (= $[M]_o$), depending on E. Since the value is sufficiently smaller than K_M (= 300 μM) for the reaction between the reduced MADH and PES^+,[23] we may suppose that both the forward and backward reactions of MADH and the mediators used are in second-order kinetics under our experimental conditions. In addition, $[M_{ox}]$ and $[M_{red}]$ are constant in the main column due to the rapid electrolysis of the mediators. Therefore, the reaction kinetics is reasonably considered as pseudo-first order, and the rate equation can be solved[14] as follows under the initial condition of $[P]_0$ ($\equiv [P_{ox}] + [P_{red}]$) = $[P_{ox}]_{t=0}$

$$\theta = \frac{\exp\left[\dfrac{-k_f(1+\eta_P)[M]_0 t}{1+\eta_M}\right] + \eta_P}{1+\eta_P} \qquad (3)$$

where θ is the fraction of P_{ox} and can be evaluated from the background-corrected absorbance of the protein (A) at the peak top and at a given wavelength as follows

$$\theta \equiv \frac{[P_{ox}]}{[P]_o} = \frac{A - A_{red}}{A_{ox} - A_{red}} \qquad (4)$$

where A_{ox} and A_{red} denote the background-corrected absorbance of the fully oxidized and reduced protein at the peak top, respectively. η_P is the ratio of the equilibrated concentration of P_{ox} and P_{red}, as defined by

$$\eta_P \equiv \frac{[P_{ox}]_{eq}}{[P_{red}]_{eq}} = \exp\left[\frac{2F}{RT}(E - E^{o\prime}_P)\right] \qquad (5)$$

where $E^{o\prime}_P$ is the formal redox potential of the protein.

In this work, the θ values were evaluated from A at 331 nm according to eq 4, where A_{ox} and A_{red} were obtained from the FIA peaks of MADH at $E \gg E^{o\prime}_P$ and $E \ll E^{o\prime}_P$, respectively, at a given f. Figure 4 shows the t dependence of θ at various E values.

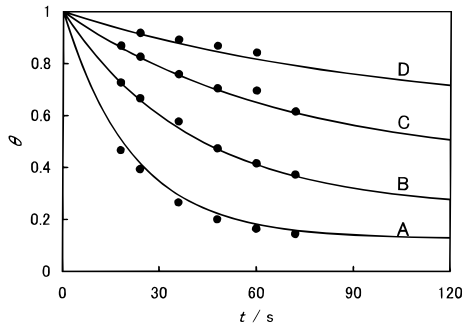

Figure 4. Dependence of the fraction of the oxidized form of MADH (θ) on the reaction time (t) at E = (A) −0.090 V, (B) −0.080 V, (C) −0.070 V, and (D) −0.060 V in the presence of PMS^+ ($E^{o\prime}_M$ = −0.132 V) at 50 μM (= $[M]_o$). The solid lines represent the regression curves obtained by nonlinear analysis on the basis of eq 3 with $E^{o\prime}_P$ = −0.065 V and $k_f = 2.1 \times 10^4$ M^{-1} s^{-1}.

Thus, we tried to fit eq 3 to the θ vs t plots by means of a nonlinear least-squares method using $E^{o\prime}_P$ (or η_P) and k_f as adjustable parameters. Evaluated values of $E^{o\prime}_P$ and k_f were −0.065 ± 0.002 V and (2.14 ± 0.08) × 10^4 M^{-1} s^{-1}, respectively, in the PMS^+ system.

Similar experiments were performed using PES^+ (50 μM) or PMS-S (50 μM) as a mediator in place of PMS^+. In both cases, no spectral signal of the semiquinone intermediate of TTQ in MADH was observed. The spectral data of the FIA peaks were analyzed in the same manner as described above. The nonlinear regression results were (1) $E^{o\prime}_P$ = −0.072 ± 0.001 V and k_f = (1.37 ± 0.08) × 10^5 M^{-1} s^{-1} in the case of PES^+ and (2) $E^{o\prime}_P$ = −0.059 ± 0.001 V and k_f = (6.2 ± 0.1) × 10^2 M^{-1} s^{-1} in the case of PMS-S. The $E^{o\prime}_P$ values evaluated using the three mediators are close with each other, and the averaged value was −0.065 V with a standard deviation of 0.005 V.

Although the number of the data points is limited, the logarithmic value of k_f seems to increase with a decrease in $E^{o\prime}_M$, as expressed by log (k_f) = − 21 $E^{o\prime}_M$ + 1.7 or log(k_f) = −0.62(2F/2.303RT)($E^{o\prime}_M$ − $E^{o\prime}_P$) + 3.1 (r = 0.98). Such a linear free energy relationship has been reported in the reversible electron-transfer reaction between diaphorase and quinones.[24] Interestingly, the slope and the intercept of the linear relation of the diaphorase/quinone system are close to those obtained here. There are also several examples of the linear free energy relationship in the oxidation of proteins with mediators.[25−29] Such a property seems to be useful for selection of mediators.

Equation 3 also suggests that $E^{o\prime}_P$ can be evaluated from the $[M]_o$ dependence of θ at a given t. The data are plotted in Figure 5 for the PMS^+ system. The regression analysis of the data yielded $E^{o\prime}_P$ = −0.071 ± 0.008 V and k_f = (1.9 ± 0.1) × 10^4 M^{-1} s^{-1}, which are close to those obtained from the θ vs t relationship

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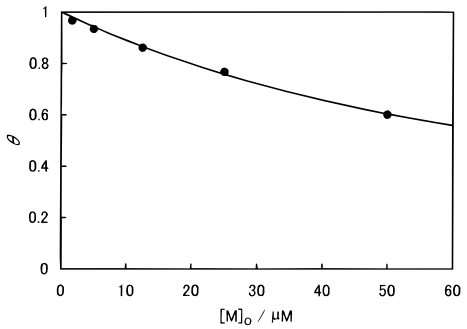

Figure 5. Dependence of the fraction of the oxidized form of MADH (θ) on the concentration of PMS^+ (= $[M]_o$) at $E = -0.080$ V and $t =$ 36 s. The solid line represents the regression curve obtained by nonlinear analysis on the basis of eq 3 with $E^{o\prime}_P = -0.071$ V and $k_f = 1.9 \times 10^4$ M^{-1} s^{-1}.

(Figure 4). These results support the idea that the kinetic analysis on the mediated CFCESET is effective for the determination of $E^{o\prime}_P$.

Kinetic Aspects in Thermodynamic Analysis. Generally, it would be fairly difficult to attain the equilibrium between a protein and a mediator in a short time. The half-life time ($\tau_{1/2}$) in eq 3 is given by

$$\tau_{1/2} = \frac{\eta_M + 1}{k_f[M]_o(\eta_P + 1)} \ln 2 \qquad (6)$$

In the case of the MADH/PES^+ system where $E^{o\prime}_M < E^{o\prime}_P$, $\tau_{1/2}$ becomes $\ln 2\eta_M/(k_f[M]_o\eta_P)$ {= $\ln 2 \exp[(2F/RT)(E^{o\prime}_P - E^{o\prime}_M)]$} at $E > E^{o\prime}_P$ ($\eta_M \gg \eta_P \gg 1$) and $\ln 2/(k_f[M]_o)$ at $E < E^{o\prime}_M$ ($\eta_M \ll \eta_P \ll 1$). Then it takes a longer time to reach the equilibrium at $E > E^{o\prime}_P$. The opposite situation is encountered for the MADH/PMS-S system ($E^{o\prime}_M > E^{o\prime}_P$). Such E dependence of $\tau_{1/2}$ is depicted in Figure 6 as curves A and B.

When we use more than one mediator (M_1, M_2, ..., M_j) in the mediated CFCESET, the rate equation of the one-mediator system (eq 3) can be rewritten as

$$\theta = \frac{\exp\left[-\sum\left(\frac{k_{f,j}[M_j]_o}{1+\eta_{M,j}}\right)(1+\eta_P)t\right] + \eta_P}{1+\eta_P} \qquad (7)$$

where $k_{f,j}$ and $\eta_{M,j}$ represent k_f and η_M of M_j, respectively. The $\tau_{1/2}$ in this system is given by

$$\tau_{1/2} = \frac{\ln 2}{(1+\eta_P)\sum\left(\frac{k_{f,j}[M_j]_o}{1+\eta_{M,j}}\right)} \qquad (8)$$

Figure 6, curve C shows the E dependence of $\tau_{1/2}$ calculated for MADH in the two-mediator system consisting of PES^+ and PMS-S. In this calculation, the kinetic and thermodynamic parameters evaluated in the above sections were used, and the total concentration of the mediator was set as equal to that of the one-mediator system (curves A or B). Figure 6 clearly proves that

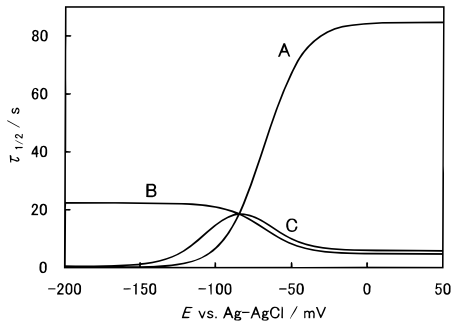

Figure 6. Half-life time ($\tau_{1/2}$) of the MADH/mediator reaction system as a function of the electrode potential (E). Mediator: (A) PES^+ ($E^{o\prime}_M = -0.155$ V, $[M]_o = 50$ μM), (B) PMS-S ($E^{o\prime}_M = -0.048$ V, $[M]_o = 50$ μM), (C) PES^+ ($[M_1]_o = 10$ μM) + PMS-S ($[M_2]_o = 40$ μM). The solid lines were calculated on the basis of eq 6 (for curve A and B) or eq 8 (for curve C) using the following parameters: $E^{o\prime}_P$ (for MADH) = -0.068 V, $k_{f,1}$ (for PES^+) = 1.4×10^5 M^{-1} s^{-1}, $k_{f,2}$ (for PMS-S) = 6.2×10^2 M^{-1} s^{-1}.

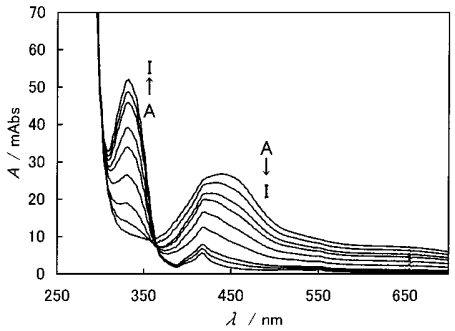

Figure 7. Background-corrected absorption spectra of the FIA peak of MADH (50 μM × 10 μL) in the presence of 10 μM PMS^+ and 40 μM PMS^+ PMS-S at $t = 144$ s. E = (A) 0 V, (B) −0.035 V, (C) −0.050 V, (D) −0.060 V, (E) −0.070 V, (F) −0.080 V, (G) −0.095 V, (H) −0.110 V, (I) −0.140 V.

the use of two mediators with $E^{o\prime}_M$ more negative and positive than $E^{o\prime}_P$ (PES^+ and PMS-S in this case) is convenient to reduce $\tau_{1/2}$ in the wide potential range. The maximum $\tau_{1/2}$ value is about 18 s around $E^{o\prime}_P$ at the conditions of $[PES^+]_o = 10$ μM and $[PMS\text{-}S]_o = 40$ μM. The time required to reach the equilibrated state will be at most 110 s ($\approx 6\tau_{1/2}$), which is shorter than the maximum column electrolysis time ($t = 200$ s).

To verify this point, the mediated CFCESET was employed using PES^+ and PMS-S as mediators to obtain the thermodynamic data. As expected from the above kinetic consideration, the experimental θ values of the MADH peak at $[PES^+]_o = 10$ μM and $[PMS\text{-}S]_o = 40$ μM were independent of f in the range from 0.6 to 0.4 mL min^{-1} ($t = 120$−180 s). This result indicates that the reaction between MADH and the mediators has reached the equilibrated state at least $t > 120$ s. Figure 7 shows the absorption spectra of the equilibrated MADH at various E values under the conditions of $t = 144$ s ($f = 0.5$ mL min^{-1}). The spectra yielded an isosbestic point at 363 nm, indicating one-step two-electron transfer without generation of the semiquinone intermediate of MADH.

We tried curve fitting to the plots of the A value at 331 nm against E using the following equation

$$A = (\epsilon_{P,ox}[P_{ox}] + \epsilon_{P,red}[P_{red}])l$$
$$= \frac{(\epsilon_{P,ox}\eta_P + \epsilon_{P,red})[P]_o l}{\eta_P + 1} \quad (9)$$

The regression analysis yielded $E^{o\prime}_P$ of MADH as -0.068 ± 0.001 V, as shown in Figure 8. This result was in good agreement with the evaluated value from the kinetic analysis described above. This agreement strongly supports the validity of the kinetic analysis on the mediated CFCESET for the measurement of the redox potential. However, the evaluated value of the redox potential of MADH was slightly more positive than the reported one (-0.10 V at pH 7.5) obtained by the mediated OTTLSET[22] and potentiometric-spectroscopic titration[30] using PMS^+ and PES^+ as mediators. The reason for the discrepancy was unclear. The kinetic approach is also valuable for optimizing the conditions for attaining the equilibrium between protein and mediators and for ensuring the equilibrium of the system.

CONCLUDING REMARKS

This work has clearly shown the significance of the kinetic approach in the mediated CFCESET for determining the redox potential of proteins, where it is not necessarily required to attain the equilibrium during the indirect column electrolysis. This situation makes it easy to select mediators. A variety of mediators can be used as long as both of the fully reduced and oxidized states of proteins are observed at $E < E^{o\prime}_P$ and $E > E^{o\prime}_P$, respectively, in the mediated CFCESET. The kinetic analysis makes it simple to deduce the thermodynamic and kinetic parameters.

Parker and Seefeldt tried simulating a cyclic voltammogram in the mediated OTTLSET to get the kinetic and thermodynamic parameters of protein redox reactions.[31] However, the mass-transfer effect is completely neglected in their analysis, which would cause erroneous results in the OTTLSET. Kasmi et al. proposed another kinetic analysis for the determination of the potential of a redox enzyme from the steady-state enzyme kinetics.[32] Their analysis, however, is based on the assumption that the enzyme exists in the redox equilibrium before and during the enzymatic reaction. It seems to us impossible to have such a situation. Our kinetic approach is more rigorous and reliable.

Generally speaking, the thermodynamic analysis would be superior to the kinetic analysis for the determination of protein redox potentials. In the thermodynamic analysis in the indirect methods, however, it is essential to ensure that the system is in equilibrium. The only way to do so would be to judge whether the signal becomes time-independent. However, practically speaking, such criterion of the time-independent characteristics would be determined by workers themselves and be ambiguous. In the mediated CFCESET, the change in θ of the redox process can be well characterized as expressed by eq 3 or 7. Therefore, the criterion for the equilibrium would be clearly described in terms of the half-life time. Such theoretical argument would be difficult in other indirect measurements including the mediated OTTLSET, in which the spectral change after the potential step is ascribed to both the mass-transfer-involved electrode process of mediator and the homogeneous redox process between protein and mediator. In conclusion, the kinetic analysis and/or aspect in the mediated CFCESET are very useful for the determination of the redox potential of proteins.

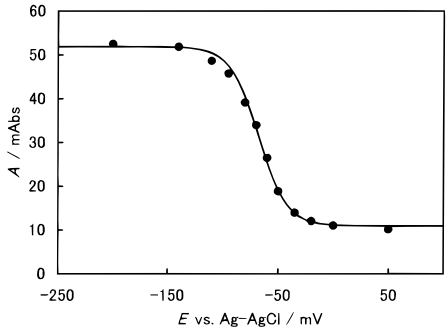

Figure 8. Background-corrected absorbance (A) of the MADH peak at 331 nm as a function of the electrode potential (E). The data were obtained under the conditions of Figure 7. The solid line represents the regression curve obtained by nonlinear analysis on the basis of eq 9 with $E^{o\prime}_P = -0.068$ V.

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