Determination of the Molar Absorption Coefficients of *c*-Type Cytochromes Using Optically Transparent Thin-Layer Electrodes

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The simultaneous monitoring of both the absorbance and charge consumed in electrolysis in a precalibrated Optically Transparent Thin-Layer Electrode (OTTLE) cell was demonstrated as a precise method for determining the molar absorption coefficients of electron-transfer proteins. In this method, the procedure for a direct determination of the concentration of the protein could be omitted. The values of the molar absorption coefficients of *c*-type cytochromes at the α-peaks of reduced forms were determined by this method: 28.4 $mM^{-1}\,cm^{-1}$ (1 M=1 $mol\,dm^{-3}$) at 550 nm for horse heart cytochrome *c*, 119 $mM^{-1}\,cm^{-1}$ at 552 nm for cytochrome c_3 from *Desulfovibrio vulgaris*, Miyazaki F, and 116 $mM^{-1}\,cm^{-1}$ at 552 nm for cytochrome c_3 from *Desulfovibrio vulgaris*, Hildenborough.

An understanding of the physicochemical properties of electron-transfer proteins has recently drawn a great deal of attention. It is very important not only to elucidate well-resolved molecular structures, but also to determine the precise values of the parameters which characterize their basic physicochemical properties. One of these parameters is the molar absorption coefficient (molar absorptivity), ε.

In order to determine the value of ε by using a conventional spectrophotometric method, the concentration of the protein of interest in a solution must be determined with a high degree of accuracy. Because of the uncertain amount of water, even in a carefully dried protein sample, weight measurements are usually erroneous. In cases where the protein molecule possesses a known number of hetero atoms, a chemical analysis of the amount of the hetero atoms has been regarded as being the best method for determining the concentration. For heme proteins, the chemical analysis methods employed in order to determine the iron content are the pyridine ferrohemochrome reaction,[1-3] titration with titanium(III) sulfate,[4] and the 1,10-phenanthroline method.[5] Also an analysis of the sulfur content[6] and potentiometric titration[7,8] were employed. These methods, however, gave rise to discrepancies among the values of ε for a particular protein. Even in the case of horse heart cytochrome *c*, which is one of the proteins most extensively studied, the reported values of ε range from 26.1 to 29.9 $mM^{-1}\,cm^{-1}$,[6-16] though these values are near to the more recent value, 29 $mM^{-1}\,cm^{-1}$ (based on iron content analysis),[17] to which a majority of papers refer. The variance among the values of ε was due to difficulties in bringing the analytical reaction or equilibrium conditions into completion reproducibly.

We hereby propose a method that makes it possible to determine ε without knowing the concentration of the proteins. In this method, both the absorbance of the solution and the charge consumed during the electrochemical reduction are monitored simultaneously. Namely, it is controlled-potential coulometry using an optically transparent thin-layer electrode (OTTLE), which is calibrated by a standard redox substance before use. This method uses charge consumed during an electrochemical reduction instead of the amount of hetero atoms as a measure of the concentration.

The spectroelectrochemical technique with OTTLE provides a simple and accurate means of characterizing the stoichiometry and thermodynamic parameters of biomolecules.[18] Controlled-potential coulometry using OTTLE has been widely utilized for measurements of the formal potential as well as the number of electrons involved in a charge-transfer reaction, but usually in the presence of an electron-transfer mediator.[18,19] Few attempts to obtain the absorption coefficients using OTTLE have been reported. Heineman and Kuwana measured the absorption coefficients of metal atoms dissolved in a thin film of mercury using a mercury–platinum optically transparent electrode.[20] Hawkridge and Kuwana measured the value of ε of cytochrome *c* in the solution phase by indirect titration at OTTLE.[21] In using their method, however, it is necessary to know the concentration of cytochrome *c*, the extent of completion of a homogeneous reaction between the mediator and cytochrome *c*, and the precise volume of the cell. Since a cell of 2.5 ml volume is employed,[21] it might not be easy to minimize the error in evaluating the above-mentioned factors.

In this paper we demonstrate the determination of the absorption coefficients of *c*-type cytochromes by using controlled-potential coulometry at an OTTLE under simultaneous monitoring of the absorbance. First, the cell-constant of an OTTLE was calibrated by using hexacyanoferrate as a standard substance, and the controlled-potential coulometry at the OTTLE was tested for a monoheme protein, horse heart cytochrome *c*. Then, the absorption coefficients of four-heme proteins, cytochromes c_3, were measured.

Experimental

Materials. Horse heart cytochrome *c* (Type VI from Sigma Chemical Co.) was purified chromatographically as described in another report.[22] Cytochromes c_3 were extracted from two different strains of sulfate-reducing bacteria, *Desulfovibrio vulgaris*, strain Miyazaki F (*Dv*MF) and *Desulfovibrio vulgaris*, Hildenborough (*Dv*H). They were purified chromatographically until their purity indices (the ratio of absorbance of the reduced form at α-peak against that of the oxidized at 280 nm) became larger than 2.95. Gold mesh minigrid (500 lines per inch, 43% transmittance) was purchased from Buckbee Mears Co. Water was purified to 16 MΩ cm through Milli Q (Millipore Co.). The other chemicals were of reagent grade and were used without further purification.

Apparatus. The OTTLE cell was constructed in nearly the same manner as Heineman has reported.[19,23] A gold minigrid-working electrode was sandwiched between two optical glass plates to form a working electrode compartment. The compartment's thickness was adjusted so that it would be in the range of 0.08—1.2 mm by spacing teflon tape between the two optical glass plates. The typical size of the gold minigrid electrode was 2.3 cm (width)×2.1 cm (height). A platinum counter electrode was dipped into the bottom compartment (approximately a 0.3 ml volume) which was connected to the reference electrode compartment through a porous Vycor tip. The reference electrode was a silver/silver chloride electrode in a saturated KCl solution (+0.197 V vs. NHE). A double-beam spectrophotometer (Shimadzu Model UV-240) was used. Employed for electrochemical measurements were a Fuso 312-S1 potentiostat, a Fuso HF-201 coulometer, and an X-t or X-Y recorder.

Procedure. The gold minigrid electrode was cleaned successively with nitric acid, acetone, and pure water in the working-electrode compartment. Then, a deaerated 1.0 M $HClO_4$ solution was used to fill the cell, and cyclic potential sweeps were carried out both to effect the oxidation-reduction cycle treatment of the gold minigrid electrode and to check the cleanness of the gold electrode by comparing the current-potential curve to the well-known characteristics of a clean gold electrode surface, as reported by Hinnen et al.[24] The position of the cathodic peak, which corresponds to the reduction of gold oxides formed during an anodic sweep, also enabled us to verify that the ohmic drop in the OTTLE cell was negligibly small. The $HClO_4$ was removed from the working electrode compartment by first washing with pure water and subsequently with a buffer solution. The OTTLE cell, enclosed in an acrylic resin box to achieve an O_2-free atmosphere, was set in the sample chamber of the spectrophotometer so that the light beam passed perpendicularly through the gold minigrid. The light beam (with a cross section of 1.2 cm×0.15 cm) was positioned near the center of the minigrid. When the OTTLE cell contained a buffer solution, the background absorbance was set at zero in the wavelength region of interest. Then, the cell was filled with a deaerated sample solution. The flow of argon gas presaturated with water into the acrylic resin box was started before the electrochemical procedures. The electrode potentials were varied stepwise. All of the experiments were conducted at 24±1 °C. All of the electrode potentials in this paper are given with respect to a Ag/AgCl electrode in a saturated KCl solution.

Results

Calibration of OTTLE Cell-Constant. The OTTLE cell was filled with a hexacyanoferrate(III) ($[Fe(CN)_6]^{3-}$) solution in 1 M KCl after deaeration. Controlled-potential coulometry was conducted between 450 and 50 mV, and the absorption spectra and charge consumed in the electrode process were recorded simultaneously. The concentration ratio of [O]/[R] (the ratio of concentration of oxidized form against that of reduced form) was calculated at each applied potential, (E), by using the equation[18]

$$[O]/[R]=(A_{red}-A)/(A-A_{ox}), \quad (1)$$

where A is the absorbance of OTTLE at a given applied potential at a wavelength of 402 nm. (At 402 nm, hexacyanoferrate (II) has an absorbance peak.) A_{red} and A_{ox} are the absorbances (at 402 nm) measured at 50 and 450 mV, where the hexacyanoferrate ions are in the fully reduced and fully oxidized forms, respectively. A Nernst plot, ln([O]/[R]) versus E, produced a straight line with a slope of −25.7±0.3 mV. This means that the number of electrons involved in the redox equilibrium (n_{eq}) was 1.0±0.01. Since the hexacyanoferrate(II/III) couple exhibits a one-electron reaction, this value ensures that equilibrium at OTTLE is established at each of the controlled potentials.

The total charge consumed by electrolysis to convert the fully reduced state into the fully oxidized state (Q_t) is given as

$$Q_t=nFC_tV, \quad (2)$$

where F is the Faraday constant, n is the number of

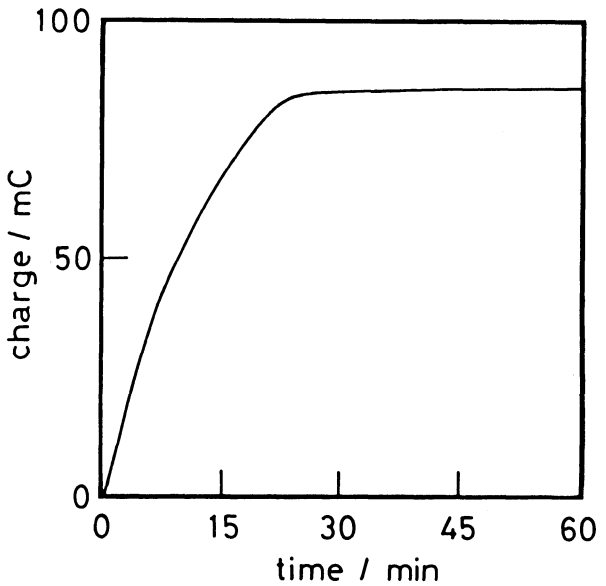

Fig. 1. Charge-time curve for a potential step from 450 to 50 mV in an OTTLE cell with a thickness of about 0.1 cm. The cell was filled with a hexacyanoferrate solution (1.8 mM) in 1 M KCl.

electrons per molecule needed to complete a redox reaction (in other words, the number of redox active centers per molecule which participate in the electron-transfer reaction in the potential range of interest), C_t is the total concentration of hexacyanoferrate, and V is the effective volume responsible for electrolysis in the OTTLE cell.

Figure 1 shows the change in the charge consumed during the electrolysis as the electrode potential was stepped down from 450 to 50 mV, where t is the time passed after the potential step. The concentration of hexacyanoferrate was 1.8 mM, and the thickness of the working electrode compartment was about 1 mm. The absorbance at the OTTLE became constant at about 27 min after the potential step. The charge was approximately proportional to $t^{1/2}$ between t=0 and 27 min, and the proportionality constant was nearly equal to the value calculated from the diffusion coefficient of hexacyanoferrate(III), according to the Cottrell equation. After t=27 min, the change in the measured charge with time became significantly small. Thus, in the volume where the gold minigrid electrode is positioned, conversion from the fully oxidized form to the reduced form is completed within 27 min. The minute change in charge after t=27 min may arise from an expansion of the diffusion layer to the volume in the vicinity of the top and bottom edges of the gold minigrid. However, as shown in Fig. 1, the charge–time curve became flat after the oxidized form was depleted in the volume, where the gold minigrid electrode is positioned. This fact indicates a minimal edge diffusion effect in the present cell configuration.[25] Thus, the value of the charge at t=27 min was taken as Q_t.

When the potential step was reversed, the same charge–time curve was obtained. The largest deviation measured over 12 runs (each 6 repetition of normal and reversal potential steps) was of the order of 2%.

The total absorbance change (ΔA) due to conversion from the fully oxidized to the fully reduced form can be written as

$$\Delta A = A_{red} - A_{ox} = \varepsilon_{red} C_t d, \quad (3)$$

where ε_{red} is the absorption coefficient of the reduced form and d is the optical path length. Note that ε_{ox} for the hexacyanoferrate(III) is zero at 402 nm and, thus, ε_{ox} is not included in the right-hand-side of Eq. 3. $Q_t/\Delta A$ is therefore equal to nFk/ε_{red}, where k is the cell constant, which is defined as V/d.

For a particular OTTLE cell, the above-mentioned measurements were carried out with various concentrations of hexacyanoferrate in a 1 M KCl solution. The relationship between Q_t and ΔA is shown in Fig. 2. In Fig. 2, Q_t and ΔA are obtained by averaging the values in 5 runs at each of the concentrations.

A least-squares fitting gave a straight line with an intercept at the origin. This fact indicates that the value of k is constant, regardless of the concentration of hexacyanoferrate. The mean value and standard deviation of k determined from the slope is 4.60±0.05 cm², using n=1.00 and ε_{red}=1023 $M^{-1}cm^{-1}$. This value of k is compatible with the apparent area occupied by the gold minigrid electrode in the OTTLE cell (2.1×2.3 cm²). This fact again indicates that the edge diffusion effect is negligible.

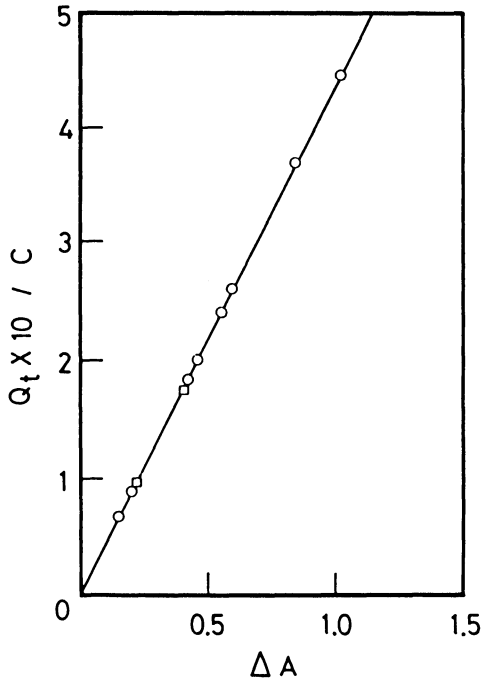

Fig. 2 Plot of Q_t, the charge needed to completely reduce hexacyanoferrate(III) to hexacyanoferrate(II), versus ΔA, the change of the absorbance at 402 nm during electrolysis in a particular OTTLE cell, at various concentrations of hexacyanoferrate (II/III) couple. The supporting electrolytes are 1 M KCl (circles), and a 30 mM phosphate buffer solution (pH 7.0) with 0.1 M NaCl (squares).

The results of measurements in a supporting electrolyte containing both a 30 mM phosphate buffer (pH 7) and 0.1 M NaCl fell on the same line obtained in a 1 M KCl solution, as shown in Fig. 2. Thus, the cell constant determined in the 1 M KCl solution can be used in the buffer solution, in which measurements for cytochromes are made.

Prior to the experiments with cytochromes, the value of k for each OTTLE cell was determined by using the procedure outlined above. The largest and typical deviations occurring in the calibration at six different OTTLE cells were 2.1% and 1.5%, respectively. Once the value of k is obtained, it is possible to calculate the value of ε for the protein of interest using this same procedure. The molar absorption coefficient of the reduced form is given by

$$\varepsilon_{red} = A_{red}/C_t d = knFA_{red}/Q_t, \quad (4)$$

where A_{red} is the absorbance measured after setting the absorbance of the OTTLE cell containing the cytochrome-free buffer solution to zero; Q_t is the value

measured for the cytochromes.

Note that the values of Q_t for the cytochromes were measured by extrapolating the linear portion of the charge–time curve to zerotime, according to a standard procedure used in thin-layer coulometry,[25–27] since the charge–time curve after depletion of the reactant in the volume where the gold minigrid electrode is positioned was not flat, but straight.

Test of the Measurement of ε for Horse Heart Cytochrome *c*. The establishment of redox equilibrium between a bare metal electrode and cytochrome *c* in the solution phase is impossible.[28] Hawkridge and Kuwana, therefore, used an electron-transfer mediator to achieve equilibrium at the OTTLE.[21] On the other hand, it is well known that at a gold electrode modified by di-4-pyridyl disulfide (4-PyS), a quasi-reversible electron-transfer reaction takes place at the formal potential of native cytochrome *c*.[29–31] In the present work, 4-PyS was used as a surface modifier in order to avoid any complication in the coulometric measurement brought about by a mediator.

The working electrode compartment of an OTTLE cell was first filled with a 4 mM 4-PyS solution in 30 mM $KClO_4$ for 10 min in order to make 4-PyS adsorbed firmly on the gold minigrid electrode surface; it was then rinsed with a 30 mM $KClO_4$ solution, and at last filled with the cytochrome *c* solution in 30 mM $KClO_4$. Controlled-potential coulometric measurements were repeated 4 to 6 times for each of the 5 different concentrations. The concentration ratio for a Nernst plot was monitored at 550 nm, which corresponds to the α-peak of the iron(II) form of cytochrome *c*. The spectra at OTTLE were the same as those measured for the cytochrome *c* solution in a quartz cuvette. A Nernst plot showed a straight line with $n_{eq}=1.01\pm0.03$ and $E^{\circ\prime}=60\pm3$ mV, where $E^{\circ\prime}$ is the formal potential. This value of n_{eq} ensured the establishment of the redox equilibrium at each of the electrode potentials. The value of $E^{\circ\prime}$ was the same as that of the native cytochrome *c*. That is, native cytochrome *c* in the solution phase was at equilibrium with the electrode and was responsible for the light-absorption at the OTTLE. The values of Q_t in the conversion between the fully oxidized (300 mV) and the fully reduced (−100 mV) states and the value of A_{red} at 550 nm were independent of the direction of the potential change or the number of repetitions. The resulting mean value of ε_{red} at 550 nm for the reduced form by using Eq. 4 was 28.4 $mM^{-1}\,cm^{-1}$ which is almost the same as the commonly used value.[17] The deviation averaged over 8 runs in total using 3 OTTLE cells was 0.5 $mM^{-1}\,cm^{-1}$, indicative of the high reproducibility of this measurement.

Measurements of ε for Cytochromes c_3 from *Dv*MF and *Dv*H Strains. Lyophilized cytochrome c_3 (*Dv*MF or *Dv*H) was dissolved in 100 mM phosphate buffer solutions (pH 7) containing 100 mM NaCl. Each of the solutions was subjected to controlled-potential coulometry using OTTLE. Cytochrome c_3 is known to be an unique class of electron-transfer proteins which can be oxidized and reduced reversibly at an electrode/solution interface without any mediators or promoters.[33] Therefore, controlled-potential coulometry at OTTLE was possible without adding any mediator or surface modifier.

For both cytochromes c_3, the absorbances at 552 nm were monitored in controlled-potential coulometry. (Both cytochromes c_3 have absorption maxima at 552 nm, which are referred to as α-peaks.) Plots of $\ln([O]/[R])$ calculated from Eq. 1 against the equilibrated electrode potentials showed apparently straight lines, resulting in the following parameters: $E^{\circ\prime}=-500$ mV and $n_{eq}=0.84$ for cytochrome c_3 of *Dv*MF, and $E^{\circ\prime}=-520$ mV and $n_{eq}=0.76$ for cytochrome c_3 of *Dv*H. These plots are not affected by the direction of the potential change.

From the macroscopic formal potentials, which were previously determined by differential pulse polarography,[34–37] the electrode potentials, where the values of [O]/[R] are unity, were calculated as −503 and −524 mV, respectively, for cytochromes c_3 from the *Dv*MF and *Dv*H strains. The values of $E^{\circ\prime}$ obtained at OTTLE by controlled-potential coulometry for both strains were in good agreement with those cited above.

The values of n_{eq} obtained at OTTLE by controlled-potential coulometry for both strains are in accordance with those calculated from the macroscopic formal potentials, as described in our previous publications.[33–36] The values of n_{eq} allowed us to reconfirm the establishment of equilibrium at each of the potentials in the present measurements.

The charge consumed in converting the oxidation states between the fully reduced (−650 mV) and the fully oxidized (−250 mV) was measured for each of the cytochromes c_3. The values of ε at 552 nm for the fully reduced forms were obtained from Eq. 4 with $n=4.00:119$ $M^{-1}\,cm^{-1}$ for cytochrome c_3 (*Dv*MF), and 116 $mM^{-1}\,cm^{-1}$ for cytochrome c_3 (*Dv*H). The average accuracy attained for these two cytochrome c_3 is ±1 $mM^{-1}\,cm^{-1}$ deviation.

Discussion

The results of the present work are summarized in Table 1.

The absorption coefficients of cytochromes per heme vary, depending on the structure or environment of the heme, but are known as being almost the same among *c*-type cytochromes.[32] The values of ε_{red} of ferrocytochromes c_3 obtained in this work are approximately 4-times as much as the value of cytochrome *c*. This result is quite reasonable, since cytochrome *c* has only one heme while cytochrome c_3 has four. Also, the values of cytochrome c_3 of *Dv*MF and *Dv*H are very close to each other. This result reflects the fact that the homology in the amino acid sequence between the two cytochromes c_3 is as high as 86.9%.[38]

Table 1. Formal Potentials ($E^{\circ\prime}$), the Apparent Number of Electrons Involved in the Redox Reaction (n_{eq}), and Extinction Coefficients at α-Peak (ε_α), as Obtained by Controlled-Potential Coulometry at OTTLE

Protein	$E^{\circ\prime}$/mV[a)]	n_{eq}	λ_α/nm	ε_α/mM^{-1}cm^{-1}
Cytochrome *c* (Horse heart)	60	1.01	550	28.4±0.5
Cytochrome c_3 (*Dv*MF)	−500	0.84	552	119 ±1
Cytochrome c_3 (*Dv*H)	−520	0.76	552	116 ±1

a) Formal potentials are given versus Ag/AgCl electrode in saturated KCl solution.

Although it was revealed that the three-dimensional structure around the four hemes is very similar between cytochromes c_3, *Dv*MF and *Dv*H,[39] most studies utilized the following values: 110 mM^{-1}cm^{-1} for *Dv*MF,[40] and 151 mM^{-1}cm^{-1} for *Dv*H.[41] A number of biophysical parameters, for example, the electron-transfer rate constant, diffusion coefficient, and dimerization equilibrium constant, could be in error if one used incorrect value of ε. The establishment of a reliable value of ε is very important, since cytochrome c_3 is presently being extensively investigated due to their unique electron-transfer properties. Recently, Hazzard and Cusanovich determined the absorption coefficient of cytochrome c_3 of *Dv*H for the 552 nm band of the reduced form species using the alkaline pyridine hemochromogen method of Flatmark[42] to be 29 mM^{-1}cm^{-1} in heme,[43] which when multiplied by 4 leads to a molecular absorption coefficient of 116 mM^{-1}cm^{-1}. Their result is in good agreement with that of this work. It is certain that the value of ε obtained in this work for cytochrome c_3 of *Dv*H is more reliable than the values found in previous studies.

A remarkable feature of the method used in the present work is that it is unnecessary to know the concentration of the protein of interest. In other words, the calibration of the OTTLE cell with a standard redox couple and a subsequent coulometric measurement, enabled us to determine ε using the charge consumed in the electrochemical reaction as a measure of the concentration. The prerequisites for an electron-transfer protein in the use of this method are: (i) the protein is soluble and able to be highly purified, (ii) the color change upon a redox reaction is detectable in the UV-vis region, (iii) a chemically reversible electron-transfer reaction occurs at OTTLE without using an electron-transfer mediator, and (iv) the redox equilibrium between the protein in the solution and the electrode can be attained without any disturbance due to the electrode reaction of the solvent.

The advantages of this method using OTTLE (as opposed to the method used by Hawkridge and Kuwana[21]) are: (i) the geometrical uncertainty of the OTTLE cell was excluded by the calibration procedure before use, (ii) completion of the reaction can be verified by the standard procedure of thin-layer cell electrochemistry,[25–27] and (iii) changes in both the charge and absorbance are only responsible for the sample of interest, since no electron-transfer mediator is used.

Conducting a redox reaction in the OTTLE cell does nothing harmful to the sample, even if the oxidation-reduction is repeated. Compared to methods using the analytical reactions or a mediator in the OTTLE cell, the sample used in the present method can be used again in subsequent studies without any isolation procedure. These features are also noteworthy, especially when the biomolecules used are of limited amount.

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