A Kinetic Study of Cytochrome *c* Adsorption to Hydrophilic Glass by Broad-Band, Time-Resolved Optical Waveguide Spectroscopy

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The adsorption process of cytochrome *c* from aqueous solution onto glass and its desorption process from glass into water were investigated by broad-band, time-resolved optical waveguide spectroscopy. The adsorption and desorption rate constants as well as the adsorption free energy of cytochrome *c* were determined by fitting the Soret-band absorbances measured at different adsorption and desorption times to the Langmuir isotherm. The surface coverage of immobilized protein molecules at equilibrium was calculated on the basis of the waveguide theory.

Introduction

Optical waveguide (OWG) spectroscopy has been recognized as a rather new and powerful technique for label-free detection of chemical and biological molecules immobilized on the waveguide surface.[1−8] OWG spectroscopy makes use of the evanescent field, which penetrates less than a wavelength out of the waveguide surface, to respond to absorption of immobilized molecules over a broad spectral bandwidth. The technique can provide much information such as the amount, orientation, and conformation of the adsorbate at the liquid/solid interface as well as the adsorption kinetics of the analyte. In our laboratory, OWG spectroscopy was developed several years ago[4] and used for investigation of the interfacial behaviors of organic dyes including methylene blue (Mb), bromothymol blue (BTB), and copper tetra-*tert*-butylphthalocyanine ($CuPt^t$).[5−7] In these earlier studies, potassium ion-exchanged (PIE) slide glass waveguides were used as transducers. PIE waveguides were robust, low-loss, and easy to fabricate, which facilitated our experiments to a certain extent. However, PIE waveguides have a narrow spectral window in the visible region, which makes us unable to effectively examine the adsorption process of hemoproteins having a strong absorption at about 410 nm (the Soret band).[4−7] Recently, we successfully improved the OWG spectrometer by use of commercially available glass plates with several tens of micrometers thickness as an alternative of PIE waveguides.[8] Such thin glass plates serving as the substrate-free multimode waveguides have several advantages such as a low loss (<1.2 dB/cm), a broad-band transparency (from 360 to 800 nm), an easy coupling without prisms, and a large sensitivity to monolayer absorption in contrast with that of PIE waveguides. With the use of horse heart cytochrome *c* as the analyte, this study demonstrates the outstanding applicability of the improved OWG spectrometer for characterizing interfacial behaviors of hemoproteins.

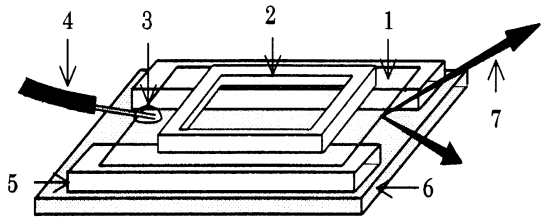

Figure 1. Schematic of the instrument used for OWG spectroscopy: 1, the 30 μm thick glass plate; 2, the silicone rubber cell; 3, the glycerol drop; 4, the glass fiber; 5, the silicone rubber strip; 6, the slide glass supporter; 7, the output light beam to be detected.

Experimental Section

Figure 1 schematically shows the OWG spectrometer used here. The apparatus includes a 30 μm thick glass plate (MATSUNAMI Glass Industry, Ltd., Japan) that is mounted to a pair of silicone rubber strips supported on a bulk glass substrate. Light from a xenon-arc lamp (150 W) passing through a glass fiber (200 μm in diameter with a 100 μm core) is coupled into the thin glass plate between two rubber strips, being 5 mm wide, by touching the fiber end to the plate and then covering the end with a drop of glycerol. One of both light beams emitted out of an endface of the glass plate was monitored by using an optical multichannel analyzer (C7473, HAMAMATSU Photonics K.K., Japan) that is capable of recording the signal once per second. A silicone rubber sheet containing a 2 cm long hole as the sample cell is attached to the glass plate. Detailed information on the instrument is available in ref 8.

Cytochrome *c* is a well-characterized biological redox protein. The protein is a spherical molecule with a 3.1 nm diameter and has a molecular weight of 12400. Cytochrome *c* containing hydrophilic groups on its outer surface is soluble in water and positively charged in the case of solution pH below 10 (the

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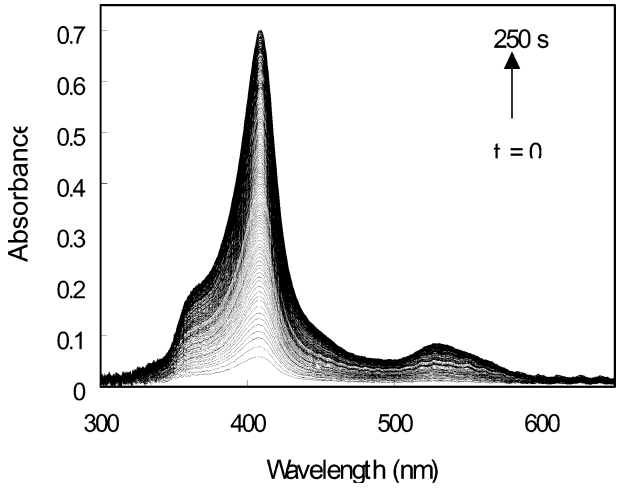

Figure 2. A total of 237 absorption spectra measured in the adsorption process of cytochrome *c* from the 1 μM aqueous solution onto the hydrophilic glass plate. The time at which the sample was introduced into the cell was given as zero ($t = 0$).

isoelectric point). The reported works show that cytochrome *c* is easy to adsorb to hydrophilic glass from aqueous solution due to electrostatic attraction (the OH groups immobilized onto glass make the surface negatively charged).[10−12] Horse heart cytochrome *c* was purchased from Sigma Chemical Co. and used as received. A 1 μM aqueous solution of cytochrome *c* was prepared using deionized water as the solvent. The solution was measured to have a pH of ca. 5. The electrophoresis confirmed that in the solution the protein carried positive charges. To investigate cytochrome *c* adsorption to glass by OWG spectroscopy (the thin-film glass plates without any surface modification are hydrophilic), deionized water is first introduced into the cell for collection of a reference spectrum. The sample spectra are obtained at different times after the water in the cell is replaced with the solution sample. The resulting absorption spectra are derived from the equation $A = \log(I_R/I_S)$, where I_R and I_S are light intensities, at a fixed wavelength, in the reference and sample spectra. After the equilibrium of the system, the solution sample is removed from the cell and the cytochrome *c* adlayer is then covered with deionized water for examining the desorption process of immobilized protein molecules by OWG spectroscopy. The experiment was performed at room temperature (~25 °C).

Results and Discussion

Figure 2 shows 237 absorption spectra obtained 250 s after the sample injection (the time interval for recording two adjacent spectra is ~1 s). The spectra include two bands, and the band at 409 nm is attributed to the Soret-band absorption of immobilized protein molecules. The empty circles in Figure 3 show the absorbances at 409 nm versus the adsorption time. The absorbance increases with time, indicating increased numbers of immobilized protein molecules. It takes more than 200 s to reach equilibrium, at which the absorbance is approximately equal to 0.7. With deionized water to cover the cytochrome *c* adlayer, the absorbance at 409 nm was observed to slowly decrease with time, indicating a slow desorption of protein molecules from glass into water.

The adsorption and desorption behaviors of cytochrome *c* were found to approximately obey the Langmuir isotherm. According to the Langmuir model,[9] adsorption of molecule M from the bulk solution onto the interface can be expressed by

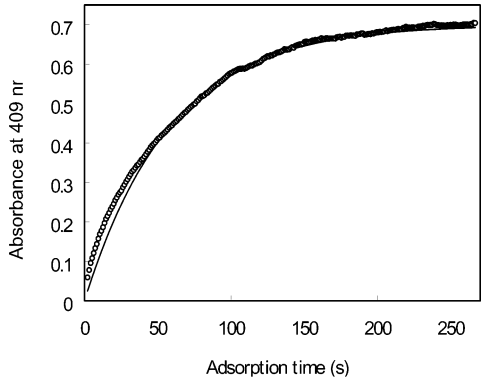

Figure 3. Absorbances, at 409 nm, of the cytochrome *c* adlayer as a function of the adsorption time (the empty circles). The black line represents the best fit to the Langmuir isotherm.

the following reaction:

$$M + S_E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} M{-}S_F \tag{1}$$

where S_E and S_F represent the empty and filled surface active sites and k_1 and k_2 are the adsorption and desorption rate constants. From reaction 1 the following kinetic equation can be derived:

$$\frac{dN}{dt} = k_1\frac{C}{55.5}(N_{max} - N) - k_2N \tag{2}$$

where C is the bulk concentration, N and N_{max} are the surface coverage (mol/cm^2) and its maximum, and 55.5 is the water molarity. Note that in eq 2 the adsorption-induced decrease in C is neglected. From eq 2 N at a fixed adsorption time t_{ad} can be determined to be

$$N = N_0(1 - e^{-tad/\tau}) \tag{3}$$

$$N_0 = N_{max}\frac{C}{(C + 55.5k_2/k_1)} \tag{4}$$

where N_0 is the surface coverage at equilibrium and $\tau^{-1} = k_1C/55.5 + k_2$. For the desorption process where C is given as zero, N as a function of the desorption time t_d can be written as follows:

$$\ln(N) = \ln(N_0) - k_2t_d \tag{5}$$

The pseudo-Beer law used below indicates that the OWG absorbance A measured at a fixed time is proportional to N at this time. Therefore, eqs 3 and 5 still hold after N and N_0 are substituted with A and A_0 (A_0 is the absorbance at equilibrium). Using the measured values of A at 409 nm for the water-covered protein adlayer, ln(A) versus the desorption time is plotted in Figure 4, where the straight line represents a linear regression. From the straight line k_2 is derived to be 1.47×10^{-4} s^{-1}. The black line in Figure 3 is the best fit to eq 3, from which τ^{-1} is determined to be 0.017 s^{-1}. With the values of τ^{-1}, k_2, and C ($C = 1$ μM), k_1 is calculated to be equal to 9.41×10^5 s^{-1}. The adsorption free energy ΔG_{ad} is defined as $\Delta G_{ad} = RT\ln(k_2/k_1)$, where R and T are the gas constant and the room temperature. ΔG_{ad} is determined to be −13.37 kcal/mol for cytochrome *c* adsorption from aqueous solution onto hydrophilic glass, which is close to that reported in ref 12 ($\Delta G_{ad} = -11.8$ kcal/mol). It is indicated in ref 13 that at 25 °C and at pH 7.0 the energy change for unfolding cytochrome *c* is $\Delta G = 22.7 \pm 2.7$ kJ/mol

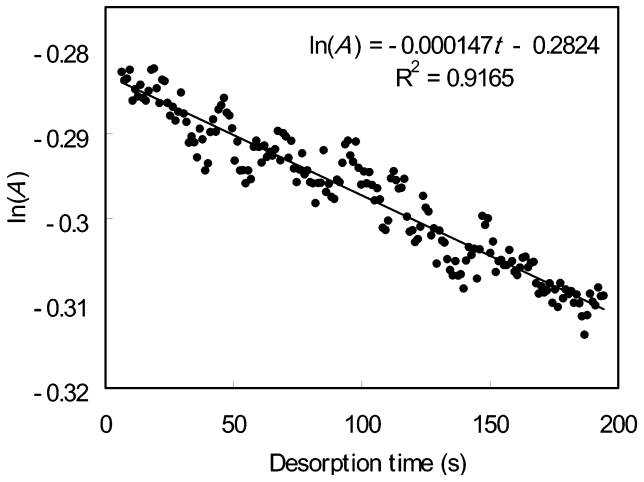

Figure 4. ln(*A*) versus the desorption time (*A* is the absorbance at 409 nm obtained with the deionized-water-covered cytochrome *c* adlayer, and the time at which the deionized water was injected into the cell was given as zero). The straight line is a linear regression.

TABLE 1: Kinetic Parameters and the Surface Coverage at Equilibrium for Adsorption of Cytochrome *c* from 1 μM Aqueous Solution onto Glass

measuring times	k_1/s^{-1}	k_2/s^{-1}	ΔG_{ad}/ (kcal/mol)	N_0/ (mol/cm^2)
1	9.41×10^5	1.47×10^{-4}	−13.37	1.29×10^{-11}
2	9.91×10^5	1.56×10^{-4}	−13.36	1.40×10^{-11}
3	9.26×10^5	1.21×10^{-4}	−13.48	1.36×10^{-11}
4	9.85×10^5	2.67×10^{-4}	−13.04	1.27×10^{-11}
5	9.00×10^5	4.56×10^{-4}	−12.67	1.22×10^{-11}

(e.g., $\Delta G = 5.43 \pm 0.65$ kcal/mol). Because the energy generated by the protein adsorption is much larger than that required for the protein unfolding, the cytochrome *c* immobilized on the hydrophilic glass surface should be denatured.

We repeated the above measurement four times using the same solution sample. The values of k_1, k_2, and ΔG_{ad} determined from each measurement are shown in Table 1. It is evident that for a specific parameter its values obtained from different measurements are quite close to each other (the small discrepancies of data between different measurements should be attributed to changes in the number of surface active sites of the thin-film glass plate used).

In addition to the kinetic parameters obtained above, OWG spectroscopy allows for the estimation of N by using the waveguide theory and the pseudo-Beer law: $A = \eta\epsilon L(N/d)$, where η is the optical power fraction in the adlayer, ϵ is the molar absorptivity, L is the interaction path length, and d is the adlayer thickness. In this study, $L = 2$ cm and $d = 3.1$ nm (the immobilized molecules are assumed to have the same size as the bulk ones). Using a conventional UV−vis spectrophotometer, ϵ for cytochrome *c* was determined to be 9.53×10^4 M^{-1} cm^{-1} at 409 nm. Because no polarizer was used in the measurements, the guided light should include the TE and TM components with equal intensities. Providing an optically isotropic adlayer of cytochrome *c*, η is the average of η_{TE} for the TE component and η_{TM} for the TM one. The guided light in the thin-film glass plate was measured to have an effective refractive index of ~1.44. On the basis of a four-layer waveguide composed of an air substrate ($n = 1$), a 30 μm glass plate ($n = 1.53$), a 3.1 nm protein adlayer ($n = 1.40$), and a water superstrate ($n = 1.33$), η_{TE} and η_{TM} at 409 nm were calculated to be 9.46×10^{-5} and 8.15×10^{-5}, respectively, in the protein adlayer. With $\eta = (\eta_{TE} + \eta_{TM})/2$ and $A_0 = 0.7$ at 409 nm, N_0 was determined to be 1.29×10^{-11} mol/cm^2, corresponding to 53% of a closely packed monolayer ($N_{max} \approx 2.4 \times 10^{-11}$ mol/cm^2). Calculations also show that N_0 is insensitive to a small change of d. For example, with $d = 2$ nm N_0 was calculated to be 1.28×10^{-11} mol/cm^2. This suggests that the calculated N_0 should not be affected by the conformational change of immobilized protein molecules. With the calculated N_0, the absorbance of the protein adlayer obtained from the conventional transmission measurement should be equal to 1.22×10^{-3} ($A = \epsilon N_0$), which is 570 times as small as that measured with OWG spectroscopy (namely, OWG spectroscopy provides a sensitivity enhancement of 570). The similar values of N_0 were obtained from different measurements (see Table 1). In addition, by use of the calculated values of $\eta_{TE} = 1.75 \times 10^{-3}$ and $\eta_{TM} = 1.67 \times 10^{-3}$ in bulk solution the bulk absorbance was obtained as 3.25×10^{-4} at 409 nm. It can be seen that the bulk solution makes a negligible contribution to the measured absorbance compared with the cytochrome *c* adlayer.

Conclusion

A kinetic study of cytochrome *c* from aqueous solution onto hydrophilic glass was accomplished by OWG spectroscopy using a thin-film glass plate as the waveguide transducer. The adsorption and desorption processes of horse heart cytochrome *c* obey the Langmuir isotherm, from which the adsorption and desorption rate constants as well as the adsorption free energy of the protein were obtained. A comparison of the energy change between two processes of the protein adsorption and unfolding suggests that on the hydrophilic glass the immobilized cytochrome *c* should be denatured. The surface coverage of immobilized protein was also determined by using the waveguide theory to calculate the optical power fraction in the adlayer. The discrepancies of the results obtained from different measurements were attributed to changes in the number of surface active sites of the thin-film glass plate used, and the sample's effect on the result discrepancies were avoided due to the use of the same solution sample. This study also demonstrates the outstanding applicability of broad-band OWG spectroscopy for characterizing interfacial behaviors of hemoproteins.

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