July, 1981] © *1981 The Chemical Society of Japan* *Bull. Chem. Soc. Jpn.*, **54**, *2189—2193 (1981)* 2189

Electrochemical Oxidation-Reduction Properties of Covalently Bound FAD of Cholesterol Oxidase Adsorbed on Mercury Electrode Surface

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(Received January 14, 1981)

Cholesterol oxidase from *Schizophyllum commune* was studied by cyclic d.c. and a.c. voltammetry with a hanging mercury drop electrode. The enzyme gave d.c. and a.c. waves due to covalently bound FAD of the enzyme adsorbed on an electrode surface. The waves were interpreted in terms of d.c. and a.c. voltammetry of a two-step surface redox reaction. The standard surface oxidation-reduction potential of the enzyme adsorbed on the electrode surface, E_s', was -0.32_5 V *vs.* SCE, the semiquinone formation constant being 1.5 ± 1.0 at pH 7.4 (25 °C). The pH dependence of E_s' was -60 mV/pH (pH 2.0—6.4), -30 mV/pH (pH 6.4—8.5), and -60 mV/pH (pH 8.5—12.0). The apparent charge transfer rate constant of the surface redox reaction was $1.6 \pm 0.5 \times 10^3$ s^{-1} at pH 5.4.

Flavoprotein oxidase is a flavin-containing enzyme which catalyzes the oxidation of a substrate by dioxygen.[1] Knowledge of the oxidation-reduction properties of this class of enzymes is important in utilizing the enzymes in electrocatalysis and for understanding the enzymatic reaction. Electrochemical methods seem to be advantageous for the study.[2] Scheller *et al.*[3] studied glucose oxidase from *Penicillium notatum* by differential pulse polarography and observed a wave with a peak at -0.34 V *vs.* SCE (pH 7.0). We[4] have studied glucose oxidase from *Aspergillus nigar* using a.c. polarography and observed a wave with a peak at -0.38 V *vs.* SCE (pH 7.0). Kuznetsov *et al.*[5] studied D-amino acid oxidase from pig kidney and observed an a.c. wave with a peak at -0.36 V *vs.* SCE (pH 7.0). In these flavoproteins FAD is bound to the apoprotein by noncovalent bond.[6] On the other hand the proteins are strongly adsorbed on the electrode surface, and thus FAD might be dissociated from the enzyme on this surface. The polarographic waves of the flavoproteins could, under certain conditions, be the waves due to the redox reaction of FAD dissociated from the enzymes on the electrode surface.

In this study we have investigated cholesterol oxidase from *Schizophyllum commune*, a flavoprotein in which FAD is covalently bound to its apoprotein,[7] by means of cyclic d.c. and a.c. voltammetry with a hanging mercury drop electrode. The enzyme gave d.c. and a.c. waves assignable to the covalently bound FAD of the enzyme adsorbed on the electrode surface. By interpreting the waves by means of cyclic d.c. and a.c. voltammetry of the two-step surface redox reaction,[8] we were able to determine the thermodynamic and kinetic parameters of the electrochemical oxidation-reduction reaction of the enzyme adsorbed on the electrode surface. The results and discussion are presented in this paper.

Experimental

Cholesterol oxidase (from *Schizophyllum commune* (ChOD), Toyobo Co., grade II, lot No. 9316) was used. This was dialyzed against 0.05 mol dm^{-3} phosphate buffer of pH 6.0 before use. The concentration of ChOD was determined spectrophotometrically, the molar extinction coefficient being 1.13×10^4 mol^{-1} dm^3 cm^{-1} at 455 nm.[9]

Voltammetric measurements were performed in the dark in an H-type cell immersed in a thermostat controlled at 25 ± 0.5 °C. The working electrode was a hanging mercury drop electrode (hmde), Metrohm E410, with surface area 0.0187 ± 0.0003 cm^2. The following buffer solutions were used: 0.1 mol dm^{-3} nitric acid–sodium acetate for pH 2.0—4.0; 0.1 mol dm^{-3} acetic acid–sodium acetate for pH 4.0—6.0; 0.1 mol dm^{-3} sodium dihydrogenphosphate–disodium hydrogenphosphate for pH 6.0—8.0; 0.1 mol dm^{-3} nitric acid–tris(hydroxymethyl)methanamine for pH 8.0—9.2; 0.1 mol dm^{-3} borax–sodium hydroxide for pH 9.2—12.0. The ionic strength of the base solution was adjusted to 0.5 mol dm^{-3} with potassium nitrate. All chemicals used were of standard reagent grade.

D.c. and a.c. voltammograms were recorded after a fresh mercury drop from the hmde had been exposed to a test solution for a given period of time, exposure time t_{exp}, and at a constant potential, initial potential E_{i}. All potentials were measured against a saturated calomel electrode (SCE). Other details on apparatus and methods of electrochemical measurements were reported.[10]

Results and Discussion

Adsorption of ChOD on Electrode Surface. Figure 1 shows a cyclic d.c. voltammogram of 0.33 μmol dm^{-3} ChOD in a solution of pH 7.4. The voltammogram was recorded after $t_{\mathrm{exp}} = 3$ min at $E_{\mathrm{i}} = -1.4$ V and with a potential sweep rate, v, 0.33 V s^{-1}. Two pairs of peak-shaped anodic and cathodic waves, ($\mathrm{I_a}$ and $\mathrm{I_c}$, and $\mathrm{II_a}$ and $\mathrm{II_c}$) and a cathodic wave ($\mathrm{III_c}$) appear at -0.33 V, -0.64 V, and -1.2 V, respectively. The waves grow to a certain limit with increasing t_{exp} at a given concentration of ChOD, c_{ChOD}. The peak heights of the waves corrected for base current, i_{p}, increase linearly with $t_{\mathrm{exp}}^{1/2}$ for small t_{exp} values, reaching a saturation value, $i_{\mathrm{p}}^{\mathrm{max}}$, for large t_{exp} values. Figure 2 shows i_{p} *vs.* $t_{\mathrm{exp}}^{1/2}$ plots for wave $\mathrm{I_c}$ at three different ChOD concentrations. The slope of the linear part of i_{p} *vs.* $t_{\mathrm{exp}}^{1/2}$ plots is proportional to c_{ChOD}. The results indicate that[10] ChOD is accumulated by adsorption on the hmde surface and the adsorption is controlled by diffusion of ChOD to the electrode surface, the current being assumed to be proportional to the amount of the adsorbed ChOD. The maximum amount of the adsorbed ChOD per unit surface area, $\Gamma_{\mathrm{ChOD}}^{\mathrm{max}}$, was determined to be $4 \pm 0.5 \times 10^{-12}$ mol cm^{-2} by means of a theoretical equation,[10] $\Gamma_{\mathrm{ChOD}}^{\mathrm{max}} = 2(D_{\mathrm{ChOD}}/\pi)^{1/2} c_{\mathrm{ChOD}} i_{\mathrm{p}}^{\mathrm{max}} t_{\mathrm{exp}}^{1/2}/i_{\mathrm{p}}$, where the diffusion coefficient of

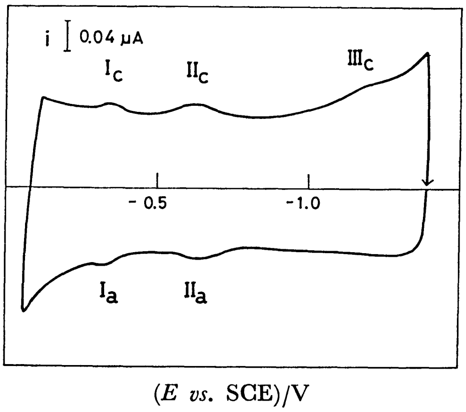

Fig. 1. Cyclic d.c. voltammogram of 0.33 μmol dm^{-3} ChOD at pH 7.4; $t_{exp}=3$ min at $E_i=-1.4$ V and $v=0.33$ V s^{-1}.

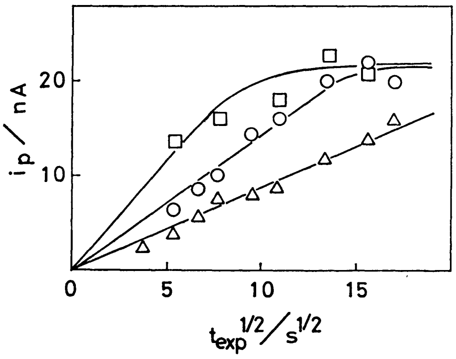

Fig. 2. Plots of i_p against $t_{exp}^{1/2}$ of waves I_c at pH 7.4. c_{ChOD}: (△) 0.24, (○) 0.38, and (□) 0.61 μmol dm^{-3}.

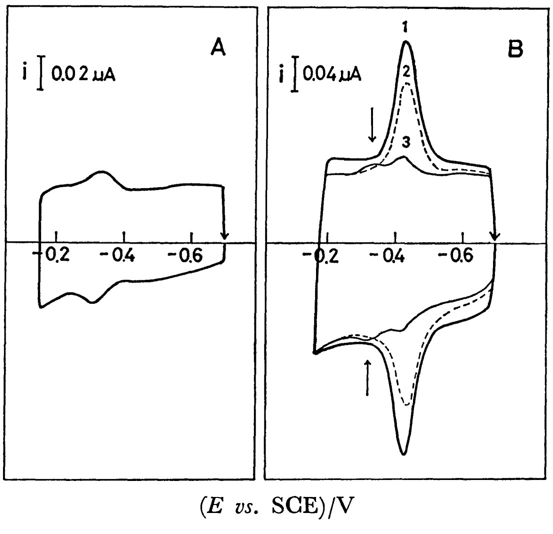

Fig. 3. (A): Cyclic d.c. voltammogram of 0.61 μmol dm^{-3} ChOD at pH 7.4, $t_{exp}=3$ min at $E_i=-0.7$ V and $v=0.10$ V s^{-1}.
(B): Cyclic d.c. voltammograms of 1.0 μmol dm^{-3} FAD and 0.14 μmol dm^{-3} ChOD at pH 7.4; $t_{exp}=$ (1) 30 s, (2) 5 min, and (3) 20 min at $E_i=-0.7$ V and $v=0.33$ V s^{-1}.

ChOD, D_{ChOD}, was estimated as 7.5×10^{-7} cm^2 s^{-1} on the basis of the diffusion coefficient of proteins[11] of about the same molecular weight as ChOD.

The following experiment gave another evidence that the voltammogram shown in Fig. 1 is due to the surface redox reaction of ChOD irreversibly adsorbed on the electrode surface. The hmde was dipped into 4×10^{-5} mol dm^{-3} ChOD solution allowing ChOD to be adsorbed on the electrode surface, washed gently with distilled water, then immersed in the same solution (Fig. 1) but containing no ChOD. The same cyclic voltammogram as in Fig. 1 was obtained.

Assigment of D.c. Waves of ChOD. The pair of waves II_a and II_c should be assigned to the half-cystine residues[9] of ChOD, since their peak potential coincides with that of the waves of apoferredoxin,[10] an SH-protein, adsorbed on the mercury electrode surface. The dependence of the peak heights, peak potentials, half-peak widths of the waves on the potential sweep rate, and that of the peak potentials on pH were explained by assigning the waves to a surface redox process,[10] $Hg+(RSH)_{ad}\rightleftharpoons(RSHg)_{ad}+H^++e^-$. The height of wave III_c increased with decrease in pH and with increase in the concentration of buffer salts of the test solutions. Riboflavin and its derivatives give catalytic hydrogen evolution current at *ca.* -1.3 V on mercury electrode in neutral and acidic solutions.[12] Thus we conclude that the wave III_c is due to catalytic hydrogen evolution by covalently bound FAD of the enzyme.

The pair of waves I_a and I_c should be attributed to the redox reaction of covalently bound FAD of the enzyme. In order to confirm that the waves are due to FAD bound to the protein and not to the free FAD which might be present in trace amount in the stock solution of ChOD, we compared the voltammogram of ChOD with that of free FAD. FAD was adsorbed on the mercury electrode surface.[13] Figure 3(B) shows cyclic d.c. voltammograms of a solution containing both 1.0 μmol dm^{-3} FAD and 0.14 μmol dm^{-3} ChOD (pH 7.4) at $t_{exp}=30$ s, 5 min, or 20 min at $E_i=-0.7$ V. FAD gives a pair of d.c. waves with the peak potential -0.43 V, 100 mV more negative than the peak potential of waves I_a and I_c (Fig. 3(A)), indicating that the latter waves are due to FAD covalently bound to the protein. The peak heights of the waves due to free FAD in the presence of ChOD decrease with increasing t_{exp}, while the waves due to ChOD begin to appear (curves 1, 2, and 3, Fig. 3(B)). This indicates that at the begining of the exposure of hmde to the test dolution the electrode surface is covered dominantly by free FAD (both the diffusion coefficient and the bulk concentration of free FAD are much larger than ChOD), but with the elapse of time the adsorbed free FAD is replaced by ChOD, probably owing to the heigher affinity of ChOD to mercury electrode than FAD.

D.c. Waves of Covalently Bound FAD of ChOD. The anodic and cathodic peak currents, i_{ap} and i_{cp}, the peak potentials, E_{ap} and E_{cp}, and the half-peak widths, $\Delta E_{a,\,p/2}$ and $\Delta E_{c,\,p/2}$, of waves I_a and I_c as a function of the potential sweep rate, v, at pH 7.4 are summarized in Table 1. Q_a and Q_c are the charges required to electrooxidize or electroreduce the

Table 1. Peak current, peak potential, half-peak width, and Integrated current of d.c. waves I_a and I_c of ChOD at pH 7.4

v / V s^{-1}	i_{ap} / nA	i_{cp} / nA	$-E_{ap}$ *vs.* SCE / V	$-E_{cp}$ *vs.* SCE / V	$\Delta E_{ap/2}$ / mV	$\Delta E_{cp/2}$ / mV	Q_a / 10^{-9} C	Q_c / 10^{-9} C
0.05	4.0	4.0	0.32	0.33	60	75	6.2	6.6
0.10	7.6	8.0	0.32	0.33	55	60	5.5	6.9
0.20	14	14	0.32	0.33	60	80	5.3	7.3
0.33	17	18	0.32	0.33	60	70	4.4	5.2

adsorbed ChOD, respectively, and estimated from the area under the waves I_a and I_c corrected for the base current. Peak heights, i_{ap} and i_{cp}, are both proportional to v, the ratio i_{ap}/i_{cp} being unity. The peak potentials and the half-peak widths are both independent of v, and E_{ap} and $\Delta E_{a,\,p/2}$ coincide with E_{cp} and $\Delta E_{c,\,p/2}$, respectively. Q_a and Q_c also coincide with each other and are independent of v. The results are in line with theoretical predictions (1) to (4), for two-step surface redox reaction which can be expressed by

$$(\mathrm{Fp.FAD^{ox}H_p})_{ad} + e^- + (q-p)H^+ \rightleftharpoons (\mathrm{Fp.FAD^{sem}H_q})_{ad} \qquad \text{I-a}$$

$$(\mathrm{Fp.FAD^{sem}H_q})_{ad} + e^- + (r-q)H^+ \rightleftharpoons (\mathrm{Fp.FAD^{red}H_r})_{ad} \qquad \text{I-b}$$

for ChOD adsorbed on the electrode surface. $(\mathrm{Fp.FAD^{sem}H_p})_{ad}$, $(\mathrm{Fp.FAD^{sem}H_q})_{ad}$, and $(\mathrm{Ap.FAD^{red}H_r})_{ad}$ denote the oxidized, intermediate (semiquinone), and reduced forms of the adsorbed ChOD, respectively. Theoretical expressions[8] of the d. c. anodic and cathodic currents in cyclic voltammetry, i_a and i_c, of the two-step surface redox reaction are given in a simplified case[14] by

$$i = i_a = -i_c = FA(F/RT)\Gamma v\rho[\rho(K^{1/2}\rho+2) + (K^{1/2}+2\rho)]/(1+K^{1/2}\rho+\rho^2)^2 \qquad (1)$$

where $\rho=\exp[(F/RT)(E-E'_s)]$, $E'_s=(E'_1+E'_2)/2$, and $K=\exp[(F/RT)(E'_1-E'_2)]$, Γ is the amount of adsorbed ChOD per unit surface area, K the semiquinone formation constant defined by $K=[(\mathrm{Fp.\ FAD^{sem}H_q})_{ad}]^2/[(\mathrm{Fp.\ FAD^{ox}H_p})_{ad}][(\mathrm{Fp.\ FAD^{red}H_r})_{ad}]$, and E'_s, E'_1, and E'_2 are the standard surface oxidation-reduction potentials of the redox couples $(\mathrm{Fp.\ FAD^{ox}H_p})_{ad}/(\mathrm{Fp.\ FAD^{red}H_r})_{ad}$, $(\mathrm{Fp.\ FAD^{ox}H_p})_{ad}/(\mathrm{Fp.\ FAD^{sem}H_q})_{ad}$, and $(\mathrm{Fp.\ FAD^{sem}H_q})_{ad}/(\mathrm{Fp.\ FAD^{red}H_r})_{ad}$, respectively. When $K\leqslant 16$, the peak currents are given by

$$i_p = i_{ap} = -i_{cp} = FA(F/RT)\Gamma v[2/(2+K^{1/2})] = (F/RT)Qv/(2+K^{1/2}) \qquad (2)$$

where $Q(=Q_a=Q_c)$ is the charge required to electrooxidize or electroreduce the adsorbed ChOD. The peak potentials are equal to E'_s:

$$E_p = E_{ap} = E_{cp} = E'_s \qquad (3)$$

and the half-peak widths are given by

$$\Delta E_{p/2} = \Delta E_{a,p/2} = \Delta E_{c,p/2} = (2RT/F)\ln[\mathrm{f}(K)] \qquad (4)$$

where $\mathrm{f}(K)$ is a function of K.[8]

The results satisfy the predictions, indicating that $E_p=E'_s=-0.32_5$ V *vs.* SCE at pH 7.4. Thus the K value at pH 7.4 was determined to be 1.8±1.1 by Eq. 2 and 1.1±0.8 by Eq. 4. The values agree, giving the average value of $K=1.5\pm1.0$ at pH 7.4. The waves I_a and I_c obtained in the pH region 2—12 satisfy the predictions by Eqs. 1—4. Figure 4(A) shows plot of $E_p(=E'_s)$ against pH; three linear relations of $E_p(=E'_s)$ against pH, the slopes being −60 mV/pH (pH 2.0—6.4), −30 mV/pH (pH 6.4—8.5), and −60 mV/pH (pH 8.5—12.0). The first and second inflection points, 6.4 and 8.5, indicate the pK of acid dissociation constants[15] of reduced and oxidized forms of ChOD adsorbed on electrode surface, respectively. Plots of $E_p(=E'_s)$ *vs.* pH of free FAD

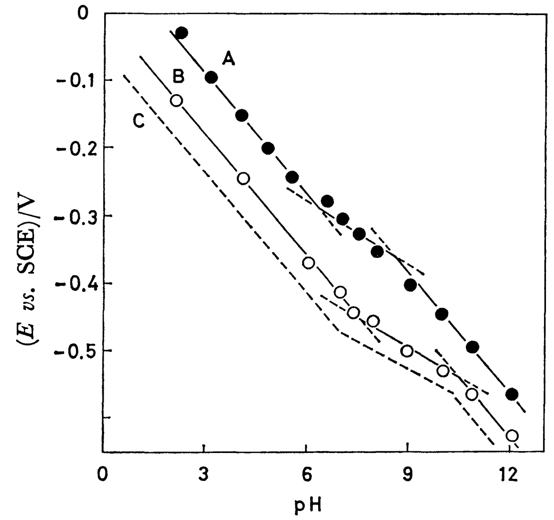

Fig. 4. Plots of $E_p(=E'_s)$ against pH; (A): ChOD adsorbed on mercury electrode surface, (B): FAD adsorbed on mercury electrode surface. (C): Broken line represents E'_m *vs.* pH curve for FAD in the bulk of solution (taken from Ref. 13).

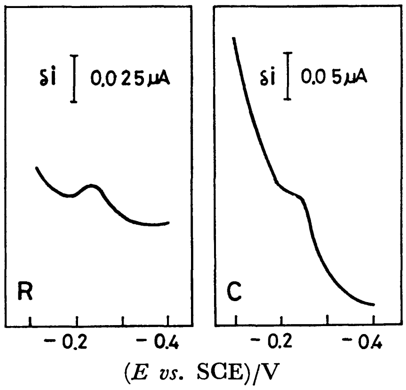

Fig. 5. A.c. voltammograms of 0.67 μmol dm^{-3} ChOD at pH 5.4 (R): Real component and (C): imaginary component at f=100 Hz; t_{exp}=3 min at E_i=−0.4 V and v=0.02 V s^{-1}.

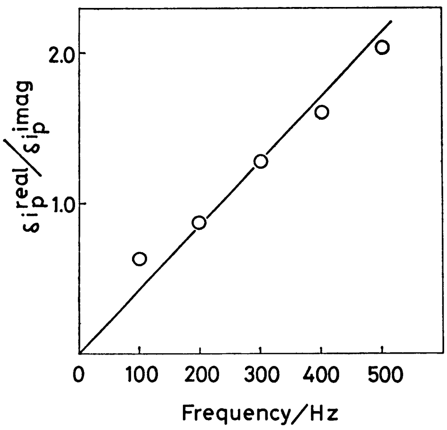

Fig. 6. Dependence of $\delta i_p^{real}/\delta i_p^{imag}$ on f for a.c. waves at pH 5.4.

adsorbed on mercury electrode surface and of the standard oxidation-reduction potential[13)] of free FAD in the bulk of solution, $E'_{m,\,FAD}$, are given in Figs. 4(B) and (C), respectively.

No standard oxidation-reduction potential of ChOD in the bulk of solution, $E'_{m,\,ChOD}$, has been determined. We tried to determine $E'_{m,\,ChOD}$ by the potentiometric titration method with dithionate as a titrant using a Pt electrode in the presence of indigo carmine la (Chroma Chemical Co., the standard oxidation-reduction potential being determined as -0.305 ± 0.015 V *vs.* SCE at pH 7.0) as a mediator. We confirmed spectrophotometrically that ChOD can be reduced by dithionate without forming a complex[16)] with sulfite, the oxidation product of dithionate. However, the system reached equilibrium only after very long time on each addition of sodium dithionate, preventing accurate determination of $E'_{m,\,ChOD}$. The $E'_{m,\,ChOD}$ value at pH 7.0 was tentatively determined to be $-0.26+0.03$ V *vs.* SCE, which is 210 mV more positive than $E'_{m,\,FAD}$ at pH 7.0. It has been suggested that covalent linkage of flavin to apoprotein shifts the oxidation-reduction potential of the flavin positively, which facilitate catalysis,[17)] a model compound hystidyl-8α-flavin being reported to have the standard oxidation-reduction potential 30 mV more positive than that of the nonsubstituted flavin.

The standard oxidation-reduction potential of a redox couple adsorbed on the electrode surface, the standard surface oxidation-reduction potential, can be shifted from the standard oxidation-reduction potential of the redox couple in the bulk of solution. This might be attributed to a difference in adsorption free energy between the oxidized and reduced species, a positive shift being observed with FMN adsorbed on mercury electrode surface.[18)] This is also the case with FAD (plots B and C, Fig. 4). In contrast, a negative shift was observed in the case of ChOD. This shift could be attributed not only to a difference in adsorption free energy but also to a change in conformation (surface denaturation) of the protein which could be associated with adsorption of the protein on electrode surface.

Table 2. Half-peak widths of real and imaginary components of a.c. wave of ChOD adsorbed on HMDE surface at pH 5.4

Frequency / Hz	$\Delta E_{p/2}^{real}$/mV		$\Delta E_{p/2}^{imag}$/mV	
	Obsd	Calcd[a)]	Obsd	Calcd[a)]
100	60	55	70	72
200	60	60	75	82
300	65	66	—	95
400	70	72	—	110
500	70	76	—	124

a) Calculated by using $K=1.2$ and $k_{sap}=1.6\times10^3\,s^{-1}$.

A.c. Waves of Covalently Bound FAD of ChOD.
In a.c. voltammetry with hmde, ChOD gave a peak-shaped wave at the potential of waves I_a and I_c in the d.c. voltammetry of ChOD with hmde. Figure 5 shows the real and imaginary component of the a.c. voltammogram of anodic scan at pH 5.4. The a.c. voltammogram of cathodic scan coincided both in height and shape with that of anodic scan. The peak potentials of the two components, E_p^{real} and E_p^{imag}, coincide with each other and with the peak potential of the d.c. wave (waves I_a and I_c) at the same pH. $E_p^{real}(=E_p^{imag})$ was independent of a.c. frequency, *f*, in the range 100—500 Hz. The ratio of the peak current of the real component to that of the imaginary component, $\delta i_p^{real}/\delta i_p^{imag}$, is proportional to f (Fig. 6). The result is in line with the prediction by a simplified equation[8,19)] of a.c. voltammograms for the two-step surface redox reaction (I-a and I-b):

$$\delta i_p^{real\prime}\delta i_p^{imag} = 2\pi f/(k_{sap}K^{1/4}), \tag{5}$$

where k_{sap} is the apparent charge transfer rate constant of the surface redox reaction. From the plot we have $k_{sap}=1.6\pm0.5\times10^3\,s^{-1}$ at pH 5.4, where $K=1.2\pm1.0$. The half-peak widths of the two components at various frequencies at pH 5.4 and their values calculated by equations[8)] using the above values of k_{sap} and K are given in Table 2. Agreement between observed and calculated values is fair.

Thermodynamic and kinetic information on the redox reaction of ChOD adsorbed on an electrode surface would be useful in designing an immobilized ChOD electrode where the electrode functions as an electron acceptor from (or electron donor to) the flavoprotein.

This work was supported by a Grant-in-Aid for Scientific Research No. 510804 from the Ministry of Education, Science and Culture.

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14) This is the case[8] when all the interaction parameters a_{ij} (i,j=ox, sem, or red) between the adsorbed reactants are of the same value. This seems a reasonable assumption since the interaction between the adsorbed protein molecules would not appreciably be affected by the change in the oxidation state of the FAD moiety of the protein because of the large size of the protein molecule. Assumption that $m/n=1$[8] is made.

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19) In addition to the assumption,[14] it is assumed[8] that $k_{sap}(1)=k_{sap}(2)=k_{sap}$ and $\alpha_n=\beta_n$, where $k_{sap}(1)$ and $k_{sap}(2)$ are respectively the rate constants of the first and second charge transfer steps and α_n and β_n ($n=1$ or 2) are the transfer coefficients for cathodic and anodic charge transfer of the n-th step, respectively.