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Behavior of Flavocoenzymes at the Gold Electrode/Solution Interface studied by Electro-optical Measurement

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The behavior of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) at the interface between the solution and a gold electrode has been studied by specular reflectivity measurement and cyclic voltammetry in 0.05 M carbonate buffer (pH 10.0). Current-potential (i—E) curves obtained for FMN and FAD showed redox peaks at about −0.5 V (*vs.* Ag/AgCl), suggesting that the isoalloxazine moieties of these compounds participate in the electron transport. The reflectivity-potential curves for these compounds measured simultaneously with the i—E curves showed a marked decrease in reflectivity in the potential region more positive than about −0.8 V. Such a trend suggests that adsorption of these compounds, as well as of their reduction products, takes place on the electrode surface. In order to obtain information about the adsorbed states of these molecules, the data were analyzed according to the theoretical treatment of McIntyre and Aspnes. The results suggest that both FMN and FAD are adsorbed with their isoalloxazine rings in contact with the electrode surface. In addition, the adenine moiety of FAD appears to come partly into contact with the surface. The reduced forms of FMN and FAD are also observed to lie in essentially the same states as their oxidized forms.

Keywords——flavin mononucleotide; flavin adenine dinucleotide; redox reaction; adsorption; gold electrode; specular reflectivity; adsorption state

Flavocoenzymes are electron-transferring coenzymes which serve as the prosthetic groups of a class of respiratory enzymes, *i.e.*, flavoproteins. Most respiratory enzymes are known to be localized on the inner mitochondrial membrane; since the enzymatic reactions are considered to occur at this biological interface, adsorption may play an important role in the appearance of enzyme activity. It would therefore be of considerable interest to investigate the behavior of flavocoenzymes at the biological interface in connection with their redox reactions. In practice, however, difficulties arise in such investigations because of the presence of various surface-active compounds in biological fluids and the structural complexity of the biosurface itself.

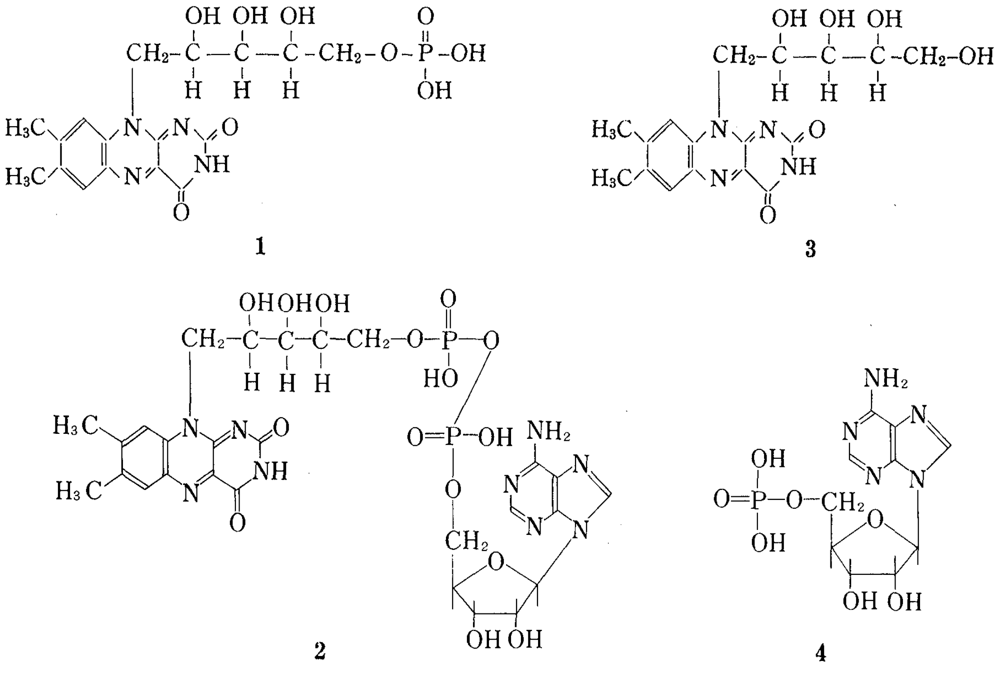
An electrode surface may be regarded as a very simple model of a biosurface, since it is a charged surface in contact with an electrolyte solution.[1] Thus, electrochemical studies of flavocoenzymes on an electrode surface may be useful for obtaining information about their biological behavior. Extensive studies on the electrochemical behavior of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) have been made.[2–8] Both FMN and FAD show a two-electron polarographic reduction wave due to two overlapping one-electron steps,[2,3] accompanied by adsorption pre- or postwave.[2,9] A number of studies relating to the adsorption have so far been carried out by potentiodynamic methods,[9-13] but little is known about the adsorbed states.[11]

The aim of the present work was to investigate the adsorbed states of flavocoenzymes in submonolayers formed on the electrode surface, in relation to their redox reactions. The specular reflectance method was employed for this purpose because of its sensitivity to the state of the electrode surface.[14] Based on the experimental results, the orientation of adsorbed species in both oxidized and reduced forms is discussed.

Experimental

Chemicals——Flavin mononucleotide (FMN) (**1**), flavin adenine dinucleotide (FAD) (**2**) and riboflavin (**3**) obtained from Wako Pure Chemical Industries, Ltd., and adenosine-5′-phosphate (AMP) (**4**) obtained from P–L Biochemicals, Inc., were used without further purification. The solutions of FMN, FAD and riboflavin were prepared immediately before the measurement. Care was taken during the entire operation to avoid photolysis of these compounds.

Sodium carbonate buffer (0.05 M, pH 10.0) was used as a supporting electrolyte solution.



1 **3** **2** **4**

Chart 1

Electrode——A gold electrode was chosen as a working electrode because of its availability over a relatively wide potential range around the point of zero charge (pzc). A gold plate of 25 mm × 22 mm was used as the working electrode. An Ag/AgCl electrode and a gold plate were used as reference and counter electrodes, respectively. Pretreatment of the working electrode was carried out by the same procedure as described in the previous paper.[15]

Measurement——All the measurements were carried out using the same apparatus as noted previously.[15,16] Electrolysis was carried out by the linear potential sweep and potential step methods. Specular reflectivity measurements were carried out during electrolysis. Perpendicularly polarized light was directed onto the working electrode surface with an incident angle of 15°. The wavelength of the light was fixed at 560 nm to avoid direct excitation of FMN, FAD and riboflavin.

During the measurements, the temperature was kept at 26 ± 1 °C. Before each run, the electrolytic solution was deaerated with purified nitrogen gas, and a nitrogen blanket was kept avove the solution.

Results and Discussion

Redox and Adsorption Behavior

Current-potential and Reflectivity-potential Curves——Current-potential (i-E) curves obtained in carbonate buffer in the absence and the presence of FMN are shown in Fig. 1.

The potential scan was started from +0.4V and reversed at −1.1V with a sweep rate of 100 mVs^{-1}. The *i-E* curve obtained without FMN shows a double layer region over the whole potential range investigated (curve a). When FMN was added to the solution (curves b and c), cathodic and anodic peaks were observed at about −0.5V. The former is attributed to the reduction of FMN and the latter to the oxidation of the electroreduction product of FMN. Both peak currents increased with increase of FMN concentration.

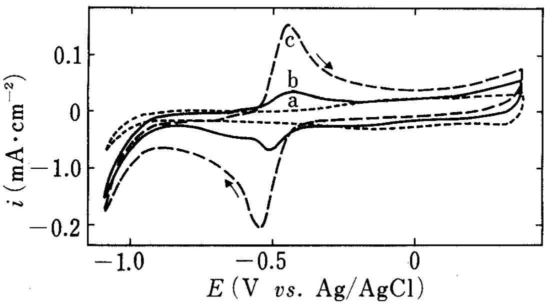


Fig. 1. Current-potential Curves obtained for FMN on a Gold Electrode in 0.05 M Carbonate Buffer Solution

FMN concentrations; a) 0, b) 5.1×10^{-5}, c) 4.8×10^{-4} M
Potential sweep rate; 100 mVs^{-1}.

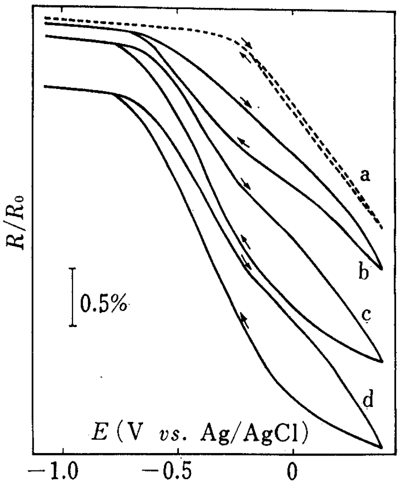


Fig. 2. Reflectivity-potential Curves obtained for FMN on a Gold Electrode in 0.05 M Carbonate Buffer Solution

FMN concentrations; a) 0, b) 5.0×10^{-6}, c) 5.1×10^{-5}, d) 4.8×10^{-4} M. Wavelength; 560 nm. Potential sweep rate; 100 mVs^{-1}.

On the *i-E* curves obtained for FAD, as well as for riboflavin, well-defined redox peaks were observed at approximately the same potentials as for FMN, suggesting that the isoalloxazine moieties of these compounds participate in the electron transport. The redox potentials are in agreement with the data for riboflavin at a gold electrode reported by Ksenzhek *et al.*[17] Since AMP itself exhibits no redox peak in the potential range investigated, it is assumed that the AMP moiety of FAD is not reduced. Thus, the reaction mechanisms for the redox reactions of FMN and FAD can be written as follows.[5,6]

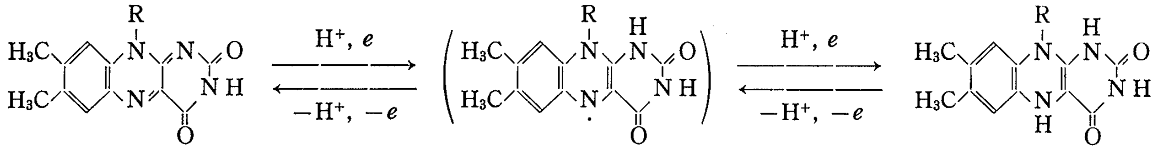


Chart 2

Reflectivity-potential (R/R_0-E) curves for FMN measured simultaneously with the *i-E* curves are shown in Fig. 2. In the absence of FMN, the R/R_0-E curve is characterized by two approximately linear portions intersecting at about −0.2 v for the sweep in either the anodic or cathodic direction (curve a). This potential corresponds to pzc for gold in moderately alkaline solution.[18] Addition of FMN causes a marked decrease in reflectivity in the potential region more positive than about −0.8 V (curves b—d, where the curves are drawn separately to avoid overlap). The reflectivity change is enhanced with increase of FMN concentration but tends to saturation and reaches the limiting value at *ca.* 5×10^{-4} M.

The adsorption of some organic compounds is known to produce a decrease in reflectivity.[15,19] In our previous studies on the adsorption of some heterocyclic compounds, the dependence of the reflectivity change on bulk concentration was found to be of Langmuir type. The hysteresis observed on the R/R_0-E curves was associated with the slow adsorption-desorption rates of the compounds.20–22) The present observations are in good agreement with these results, indicating that the adsorption of FMN takes place on the electrode surface at potentials more positive than *ca.* -0.8 V. Such changes in reflectivity are observed in the potential region either more positive or more negative than -0.5 V. This result suggests that both FMN and its reduction product ($FMNH_2$) are adsorbed on the gold electrode.

Reflectivity measurements for FAD, as well as riboflavin and AMP, were also carried out. All the results were similar to those for FMN, except for the absence of a redox reaction for AMP in the potential range investigated.

Potential and Concentration Dependence of the Reflectivity Change——To obtain the reflectivity change due to the adsorption, the reflectivity was measured potentiostatically in the same way as that previously employed.15–22) The electrode potential is first set at a certain potential where no specific adsorption of the added compound is expected, then the potential is stepped to the point at which adsorption takes place, and the R/R_0 *vs.* time curve is recorded until R/R_0 reaches its steady value. The same procedure is applied to the supporting electrolyte solution as the reference. By subtracting the latter values from the former values at the same potential, the net change in reflectivity due to the adsorption, denoted as $\Delta R/R_0$, can be obtained. In the present cases, the R/R_0-E curves in the potential region between -1.1 and -0.8 V show no change compared with those obtained for the supporting electrolyte, suggesting that none of the compounds investigated are adsorbed on the electrode surface at least in this region. Thus -1.0 V was selected as the initial potential.

The reflectivity change due to the presence of an adsorbed layer formed on the electrode surface for normal incidence has been theoretically studied by McIntyre and Aspnes based on a three-phase model.23) When the surface coverage θ is less than unity, the reflectivity change, $(\Delta R/R_0)_{\perp,\theta}$, is proportional to θ, *i.e.*,

$$\left(\frac{\Delta R}{R_0}\right)_{\perp,\theta} = \left(\frac{\Delta R}{R_0}\right)_{\perp,\theta=1} \cdot \theta \tag{1}$$

provided that the thickness of the monolayer, and optical constants of the adsorbed layer and the metal substrate are not altered during adsorption.14,23,24)

The $|\Delta R/R_0|$25) values obtained experimentally for FMN are plotted against the applied potentials in Fig. 3A. The $|\Delta R/R_0|$ value at each potential increases with increase of the FMN concentration and tends to the limiting value at sufficiently high concentration, indicating saturation of the adsorption. In the potential region from *ca.* -0.8 to -0.5 V, $|\Delta R/R_0|$ increases with increasing potential, suggesting increase in the amount of adsorbed $FMNH_2$. In the region more positive than -0.5 V, $|\Delta R/R_0|$ also increases with increasing potential and attains the maximum value at pzc, but no appreciable change is observed at more positive potentials ($|\Delta R/R_0|$ slightly decreases with increasing potential only when the concentration is very low (Fig. 3A, curve a), suggesting that the adsorbed FMN is gradually desorbed by competitive adsorption of base electrolyte anion).

The $|\Delta R/R_0|$ *vs.* E relation for FAD is plotted in Fig. 3B, in which the $|\Delta R/R_0|$ value at each potential also increases and tends to the limiting value with increasing concentration. The $|\Delta R/R_0|$ *vs.* E curves for riboflavin as well as for AMP are also shown in Figs. 3C and 3D for comparison.

Consideration of the Orientation of Adsorbed Molecules

The Oxidized Form——In order to consider the orientation of adsorbed molecules, quantitative analysis of the adsorption isotherms of FMN and FAD was attempted by the procedure described previously.16) The $|\Delta R/R_0|$ *vs.* E and $|\Delta R/R_0|$ *vs.* concentration relations suggest that no orientational change takes place at least in the potential region from -0.5 V to pzc.

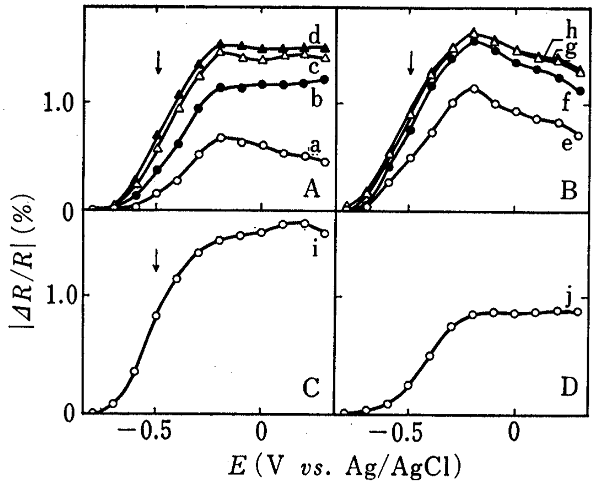


Fig. 3. Potential Dependences of the Reflectivity Changes obtained for FMN(A), FAD (B), Riboflavin (C) and AMP(D)

Concentrations; a) 1.5×10^{-6}, b) 1.5×10^{-5}, c) 1.5×10^{-4}, d) 4.8×10^{-4}, e) 1.5×10^{-6}, f) 1.6×10^{-5}, g) 1.6×10^{-4}, h) 3.5×10^{-4}, i) 1.5×10^{-4}, j) 1.4×10^{-4} M. The arrow shows the redox potential.

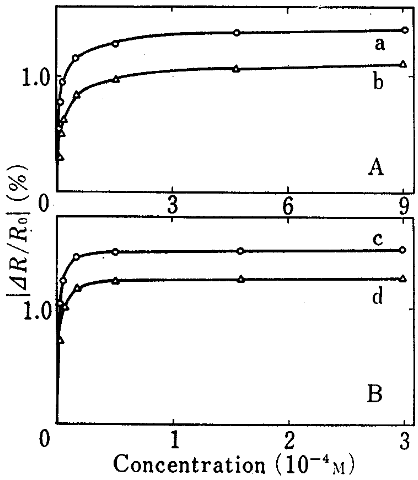


Fig. 4. Concentration Dependences of the Reflectivity Changes for FMN(A) and FAD(B) at −0.3 V (a and c) and −0.4 V (b and d).

Then, assuming the limiting value of $|\Delta R/R_0|$ at pzc to correspond to the reflectivity change at $\theta=1$, the θ values were calculated according to equation (1). The data in Fig. 4 were analyzed based on the same isotherm as employed previously,

$$\theta/(1-\theta)^p = \mathrm{cK} \qquad (2)$$

$$\mathrm{K} = \exp(-\Delta G^{\circ}_{\mathrm{ad}}/RT+\alpha F\bar{E}/RT) \qquad (3)$$

where p is the number of water molecules replaced by one molecule of adsorbed species, and c is the concentration, used in place of activity in a dilute solution. The constant K is the adsorption coefficient, $\Delta G^{\circ}_{\mathrm{ad}}$ is the standard free energy change of adsorption at pzc, α is a constant, and $\bar{E}$ is the potential difference between the pzc and the potential at which the observation is made. From the plot of $\log(\theta/\mathrm{c})$ *vs.* $\log(1-\theta)$ at a given potential, the area occupied by one adsorbed molecule of FMN or FAD on the electrode surface can be estimated in terms of p. Some examples are shown in Fig. 5, in which the plots are linear and the values of p and $\Delta G^{\circ}_{\mathrm{ad}}$ can be evaluated from the slopes and the intercepts of the lines. The adsorption parameters thus obtained for FMN and FAD are listed in Table I, together with those for riboflavin and AMP for comparison.

FMN: The p value of FMN is essentially equal to that of riboflavin, suggesting that FMN is adsorbed on the surface *via* its isoalloxazine and/or ribitol moieties. Strong adsorptivity of the ribitol moiety, however, seems unlikely because of its hydrophilic property. Assuming that an adsorbed water molecule occupies an area of 0.09 nm^2,[16] the area occupied by one adsorbed FMN is calculated to be about 0.48 nm^2. The projected area of the isoalloxazine moiety can be estimated by using a molecular model and taking into account the data on the crystal structures of riboflavin[26] and other flavin derivatives[27]; *i.e.*, 0.68—0.71 nm^2 for the flat orientation, and *ca.* 0.23 nm^2 (in contact with two methyl groups of the isoalloxazine moiety) or 0.34 nm^2 (in contact with the methyl group of C(7) and the oxygen of C(4)) for the perpendicular orientation. Comparison of the area obtained from our experiments with these values suggests that the adsorbed FMN lies on the electrode with its isoalloxazine plane inclined relative to the electrode surface. Phosphate and ribitol moieties of FMN would be directed to the solution side because of their hydrophilic property and the small repulsive interaction between the negative charge of these groups and the electrode surface. Based on the above

TABLE I. Adsorption Parameters of FMN, FAD, Riboflavin and AMP on Gold from 0.05 M Carbonate Buffer Solution (pH 10.0)

Compound	p	ΔG^0_{ad} (kj mol^{-1})
FMN	5.3 ± 0.4	-44.7 ± 1.7
FAD	6.7 ± 0.4	-62.3 ± 0.9
Riboflavin	5.1 ± 0.4	-49.3 ± 1.7
AMP	3.6 ± 0.3	-38.0 ± 0.9

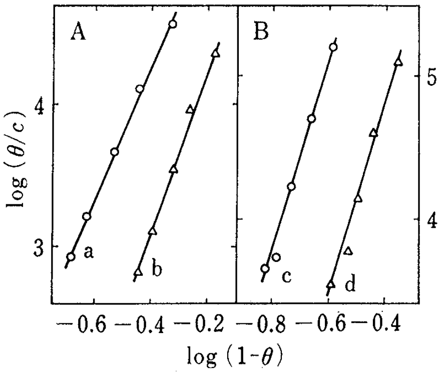


Fig. 5. Plots of log (θ/c) *vs.* log ($1-\theta$) for Adsorption of FMN(A) and FAD(B) at −0.3 V (a and c) and −0.4 V (b and d).

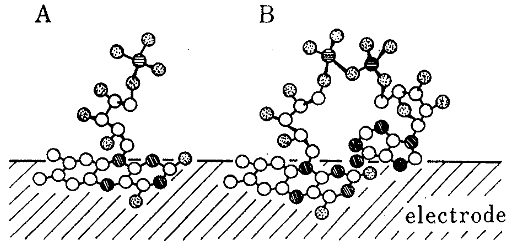


Fig. 6. Possible Models of FMN(A) and FAD(B) adsorbed on a Gold Electrode in the Potential Region from −0.5 V to pzc.

discussion, a possible model of the orientation of FMN adsorbed in the potential region from −0.5 V to pzc is schematically drawn in Fig. 6A.

In the potential region more positive than pzc, the $|\Delta R/R_0|$ *vs.* E relation (Fig. 3A) suggests that FMN remains adsorbed on the positively charged electrode surface. It has been reported that some sugars interact weakly with the electrode surface mainly at more positive potentials than pzc, especially when they are bound to phosphate groups.[28] This suggests that the presence of ribitol and phosphate groups makes the release of FMN from the electrode surface difficult.

FAD: The area occupied by one adsorbed FAD molecule in the potential region from −0.5 V to pzc was calculated to be about 0.60 nm^2 from its p value of 6.7 ± 0.4. This area is larger than those of FMN and AMP, probably implying that the FMN and the AMP moieties of FAD both participate in its adsorption. However, preferential adsorption of the FMN moiety is suggested by comparison of the ΔG^0_{ad} values of FMN and AMP.

The area of an adsorbed molecule of AMP calculated from p is about 0.32 nm^2, which is somewhat smaller than the 0.42 nm^2 planar area of adenine but is larger than the *ca.* 0.22 nm^2 perpendicular area estimated from the crystal structure.[29] Thus, AMP is regarded as being adsorbed primarily with its adenine moiety nearly flat relative to the surface. The ribose-phosphate moiety of AMP may be directed to the solution side, in analogy with the cases of deoxynucleotides.[20,21]

It seems most reasonable to consider that FAD is adsorbed with its isoalloxazine ring nearly flat relative to the surface and with a part of the adenine moiety in contact with the surface. Such an orientation is depicted by the model in Fig. 6B. The finding that the ΔG^0_{ad} of FAD is about 16 kjmol^{-1} larger than that of FMN also supports the participation of the adenine moiety in the adsorption.

In the potential region more positive than pzc, the $|\Delta R/R_0|$ *vs.* E relation is somewhat different from that for FMN. The relation (Fig. 3B) indicates that FAD is desorbed with increase in electrode potential. Its sugar (ribitol and ribose) and phosphate groups may not be able to approach the surface easily because of steric hindrance.

The Reduced Form——The same treatment as described above cannot be applied to $FMNH_2$ and $FADH_2$ in the potential region between −0.8 and −0.5 V, since $|\Delta R/R_0|$ at $\theta=1$ is difficult to obtain. The isoalloxazine moieties in these reduced forms no longer have the planar structure but a bent one as a result of hydrogenation at their N(1) and N(5) positions.[26] However, the $|\Delta R/R_0|$ *vs.* E curves are smooth over the potential range from −0.8 V to pzc (see Figs. 3A and 3B), implying that both $FMNH_2$ and $FADH_2$ are adsorbed with essentially the same orientations as their oxidized forms.

To sum up, FMN and FAD are adsorbed in both the oxidized and reduced forms on the gold electrode surface. Their isoalloxazine moieties appear principally to come into contact with the surface, and it can therefore be expected that electron transport is accomplished through these moieties.

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