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Site-Specific Immobilization of Flavin Adenine Dinucleotide on Indium/Tin Oxide Electrodes Through Flavin Adenine Amino Group

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ABSTRACT

A Mannich-type reaction was used to attach flavin adenine dinucleotide (FAD) covalently to aminosilane derivatized indium/tin oxide-coated glass plates. The aminosilane was activated with formaldehyde to give an intermediate that attached specifically to the adenine amino group of FAD. The presence of the intermediate also was demonstrated by coupling hydroquinone to the formaldehyde activated support. The immobilized FAD and hydroquinone were characterized by cyclic or differential pulse voltammetry. The immobilized FAD was shown to reduce the overpotential for NADH oxidation by 180 mV. In keeping with results for FAD on glassy carbon, FAD attached to indium/tin oxide at the adenine amino group did not lead to reconstitution of activity with apoglucose oxidase.

Index Entries: Flavin adenine dinucleotide, immobilization on In/SnO electrodes; indium/tin oxide glass; immobilization of FAD on; immobilized flavin, on In/SnO electrodes; glucose oxidase, immobilization on In/SnO electrodes; nicotinamide adenine dinucleotide, tin oxide/indium electrodes, immobilization of FAD on; electrodes, immobilization of FAD on In/SnO.

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INTRODUCTION

A wide variety of oxidation–reduction enzymes depend on the flavin isoalloxazine ring system for catalytic redox activity (*1,2*). In the past few years wide use has been made of chemically modified flavins for use in probing the active site regions of flavoenzymes (*2,3*). Such probes can provide information on several important aspects of flavin-enzyme functioning. These include, for example: (1) the three-dimensional relationship between the flavin cofactor and the apoenzyme, (2) the solvent accessability of specific sites on the cofactor molecule in the flavoprotein complex, and (3) the sites in the flavin molecule that influence the rates of electron transfer. Such information on flavin–enzyme functioning as well as novel types of chemically modified flavins can lead to eventual applications of flavins and flavoenzymes in biosensors, bioelectrochemical synthesis, and bioelectronics areas of biotechnology.

When chemically modified flavins are used to reconstitute enzymatic activity with appropriate apoenzymes, and degree of reconstituted activity is dependent on the specific site on the flavin molecule used for the modification, as well as on the size, shape, and type of attached group. The attachment of small molecular weight compounds to the flavin may result in only minor changes in enzyme activity (*3–5*). However, the attachment of an insoluble support can lead to steric interference with loss of enzymatic activity (*6*). We are interested in attaching a variety of different-sized compounds, including spacer arms and insoluble as well as soluble supports, by simple and convenient procedures.

As an outgrowth of modified flavin studies, we have been investigating the covalent attachment of flavin adenine dinucleotide (FAD) (Fig. 1) to solid electron-conducting supports. This provides an opportunity to characterize the attached flavin electrochemically while at the same time probing the active site of a reconstituted enzyme on the

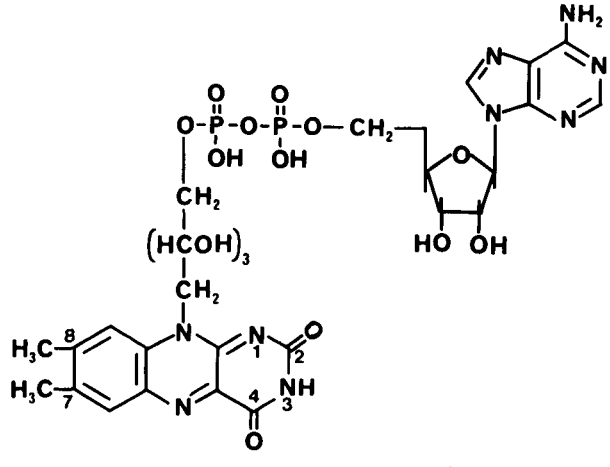


Fig. 1. Structural formula of FAD.

electrode surface. In addition, the methodology serves as a novel approach to the development of redox enzyme electrode. Two strategies are being employed. In the first strategy, it is necessary that the FAD be bound to the electrode in a way that does not preclude the reconstitution of enzyme activity upon addition of an appropriate apoenzyme. We have demonstrated some success with this approach using FAD coupled at the isoalloxazine ring position 8 to glassy carbon and reconstitution of activity with the apoenzyme of glucose oxidase (*7*). Additional work based on this first strategy is in progress with FAD and glucose oxidase as well as with other flavoenzymes. In the second strategy, the FAD is attached to the electrode surface to act as a bound mediator for the regeneration of freely diffusing cofactors, such as nicotinamide adenine dinucleotide (NAD^+), from dehydrogenase holoenzymes in solution. The rationale for this strategy arose from the observation that immobilized FAD reduced the overpotential needed for the electrochemical oxidation of NADH (*6*). It may be possible to reduce the needed overpotential even further by varying the site or method of FAD attachment to the electrode surface.

It is worthwhile mentioning other studies wherein very large molecules were covalently attached to the adenine amino group of FAD with successful reconstitution of glucose oxidase activity. One group reported 97% reconstitution of glucose oxidase activity with FAD having 40,000–60,000 molecular weight polyethyleneimine covalently attached to the adenine amino group (*8*). In a more recent study, some drug–FAD preparations, with the drug attached at the FAD adenine amino group, were capable of reconstitution of glucose oxidase activity in a few cases even with an antibody complexed to the FAD-immobilized drug (*9*). In our work with FAD coupled at the adenine amino, or possibly at a ribityl hydroxyl, group through a six-carbon spacer to glassy carbon (*6*), we were not able to reconstitute enzyme activity with the apoenzyme of glucose oxidase. Therefore, it will be important to determine what size molecules can be attached to the adenine amino function of FAD and still obtain reconstitution of activity not only with glucose oxidase but also with other flavoenyzmes.

We have concentrated our FAD electrode work on attachment through the isoalloxazine ring system (*7*) or the adenine amino group (*6*) since these represent important structural units in FAD. For the adenine amino coupling, FAD was attached covalently to carboxylic acid functionalized glassy carbon by means of a water-soluble carbodiimide (*6*). Coupling of the carbodiimide activated support most likely occurred at the nucleophilic adenine amino group of FAD; although attack on the ribityl—OH groups of FAD at a slower rate was another possibility (*10*). Therefore, we have begun to develop suitable techniques for specific coupling at FAD amino or hydroxyl groups. The method developed by Mosbach and coworkers (*11*), involving N-1 alkylation followed by Dimroth rearrangement to give N-6-modified adenine amino com-

pounds, may not be suitable for direct use on FAD. Therefore, an alternative and still easier method based on the Mannich reaction was developed.

The purpose of this paper is to demonstrate the use of the Mannich reaction for coupling of FAD to an electron-conducting support through the adenine amino group. The Mannich reaction (*12*) typically involves an amine, an aldehyde, and a material containing a labile hydrogen [HX in Eq. (1)] (*see* Fig. 3 for mechanism of reaction). Reaction of the amine and formaldehyde leads to the intermediate, **1**, shown in the iminium ion resonance form.

$$R^1R^2NH + CH_2O + HX \rightarrow R^1R^2N{-}CH_2{-}X + H_2O \qquad (1)$$

$$R^1R^2\overset{\oplus}{N}{=}CH_2$$

1

This intermediate then reacts with HX to give the final product. HX can be another amine (such as FAD), a phenolic compound (such as hydroquinone), and other compounds. However, alcoholic OH groups do not react with the intermediate (*12*). Thus, the Mannich reaction provides clear differentiation between attachment at FAD adenine amino and ribityl hydroxyl groups.

In the present work, FAD was coupled to amine derivatized indium/tin oxide (ITO) coated glass plates (*13,14*). ITO coated glass was selected as the support to examine the use of an alternative electron conducting material to glassy carbon. The attachment of FAD was documented using electrochemical techniques. Coupling of another electrochemically detectable compound, hydroquinone, was carried out, as well as enzyme reconstitution and NAD^+ regeneration tests with the immobilized FAD.

MATERIALS AND METHODS

Materials

ITO coated glass plates (NESATRON®) were obtained from Dr. James. J. Finley of PPG Industries (Pittsburgh, PA). The ITO coatings had an average surface resistance of 5–7 ohms/square. The ohms/square is defined as the specific resistance of the coating divided by the film thickness for a section of coating of equal length and width (*15*). High-purity FAD was from Boehringer Mannheim (Indianapolis, IN) as the monosodium salt. Aldrich (Milwaukee, WI) supplied γ-aminopropyltriethoxysilane, formaldehyde (37% aq.), and highest purity hydroquinone and *n*-propylamine. The silane derivative was distilled under vacuum (98–100°C/10 mmHg) p rior to use.

Preparation of ITO–FAD Plates

The ITO glass plates were cut to 5 cm long by 0.6 cm wide. They were sequentially washed with hexane, methanol, water, and methanol, with each washing accompanied by ultrasonic cleaning. Then the plates were dried in an evacuated desiccator. Silane attachment was achieved by gentle agitation of the dried plates in a solution of 10% γ-aminopropyltriethoxysilane in anhydrous toluene (v/v), which had previously been deaerated with dry nitrogen. After 1 h, the solution was decanted. The plates were washed three times with dry toluene and then vacuum dried. The terminal amino groups were activated by gentle shaking of the silanized plates in 10% aqueous formaldehyde for 1 h at room temperature. After repeated washing with water, the activated plates were used immediately for the attachment step. The formaldehyde-activated plates were immersed in 1 m*M* FAD, dissolved in deionized water, for 6 h at ambient temperature. Unbuffered solution of FAD was employed since the effects of various buffers on 2 was unknown. This was followed by repeated washing of the plates with 0.1*M* Tris buffer, pH 7.4, until FAD could not be detected in the washings by UV measurements. Control experiments were performed on plates without formaldehyde activation.

Mannich Reaction with Hydroquinone

High purity hydroquinone (1 g) was added to 4 mL deionized, deoxygenated water and solubilized with 10 drops of freshly distilled ethanol. Formaldehyde-activated ITO plates were held in this solution, under nitrogen bubbling, for 1 h in order to react with the Mannich intermediate. The plates were then washed with water and tested electrochemically for the presence of attached hydroquinone.

Electrochemical and Fluorescence Testing

Cyclic and differential pulse voltammetry were used for the electrochemical evaluations. Both techniques were carried out with a Princeton Applied Research Model 174A polarographic analyzer and a three electrode system: the working electrode, a platinum net auxiliary electrode, and a Ag/AgCl (1*M* KCl) reference electrode. The electrolyte solution was 0.1*M* Tris buffer, pH 8.0, for the experiments with attached FAD and 1*M* KCl adjusted to pH 7.0 with dilute potassium hydroxide for the hydroquinone experiments. Cyclic voltammetry was carried out at 5–50 mV/s sweep rates between +200 and −800 mV for FAD and for hydroquinone. Differential pulse voltammetry was carried out at 5 mV/s sweep rate with 50 ms long 25 mV amplitude pulses and 0.5 s intervals between pulses. All the voltammetry measurements were done under anaerobic conditions at 25°C with the plates immersed to a depth of 1 cm.

FAD-derivatized plates also were immersed in 1 m*M* NADH in 0.1*M* Tris buffer, pH 8.0, and examined by cyclic voltammetry between +1000 and −600 mV for oxidation to form NAD^+.

Fluorescence measurements of FAD were carried out with the ITO plates suspended in the cuvet of a Perkin Elmer 650-10S fluorescence spectrophotometer. Methanol was used as the solvent. Excitation was at 440 nm with emission recorded over the range 400–600 nm.

Incubation of Immobilized FAD with Apoenzyme of Glucose Oxidase

ITO–FAD-derivatized plates were incubated overnight at 4°C with the apoenzyme of glucose oxidase, prepared as described previously (*16*). Each plate was held in 5 mL of 12% aqueous glycerol, pH 7.0, containing 1.45 mg of apoenzyme. After washing away loosely adhering apoenzyme with 0.1*M* Tris buffer at pH 8.0, the plates were tested for glucose oxidase activity (*17*).

RESULTS AND DISCUSSION

Attachment of FAD to ITO Plates

Well washed immobilized FAD-ITO plates were characterized by cyclic and differential pulse voltammetry (Fig. 2). The $E^{o'}$ value, defined as midway between the reduction and oxidation peak potentials, occurred at −490 mV with reference to Ag/AgCl (1*M* KCl) for the attached FAD measured at pH 7.4 (Fig. 2A). This is very close to the value of −510 mV for FAD in solution, measured with an ITO electrode at pH 8.0, and to the $E^{o'}$ of −480 mV for FAD in solution as determined with a glassy carbon electrode at pH 8.0 (*16*); however, the $E^{o'}$ value may differ between ITO and glassy carbon electrode materials. The differential pulse voltammogram (Fig. 2B) peak of −495 mV (reference Ag/AgCl) at pH 7.4 also was similar to the differential pulse peak of −460 mV (reference Ag/AgCl) reported elsewhere for FAD at pH 8.0 (*18*). The surface loading with FAD amounted to 2–4 × 10^{-10} mol/cm^2, determined from the area under the cyclic voltammogram peaks and the geometric area of the ITO plates. This is roughly equivalent to monolayer coverage (*13*).

In control experiments, the formaldehyde activation step was omitted; and the silanized ITO plates were placed in the FAD solution and then removed and washed with deionized water. In the control experiment, initially some attached FAD was observed electrochemically. This may have resulted from adsorbed material or from ionic coupling between InO^- or SnO^- groups and protonated FAD′–NH_3^+ (where FAD′ represents FAD except for the NH_2 group), as postulated by Messing et al. for ionic coupling to glass supports (*19*). However, in the control experiments, with plates that had been continuously washed with 0.1*M*

Tris buffer at pH 7.4 and ultrasound agitation, the results indicated that the plates were free of electrochemically detectable FAD.

The cyclic voltammograms also were obtained over a range of scan rates. The results are shown in Table 1. A plot of log (peak current) versus log (scan rate) gave excellent linearity, with a correlation coefficient of 0.999. The linear-least-squares fitted slope was 0.98 for the

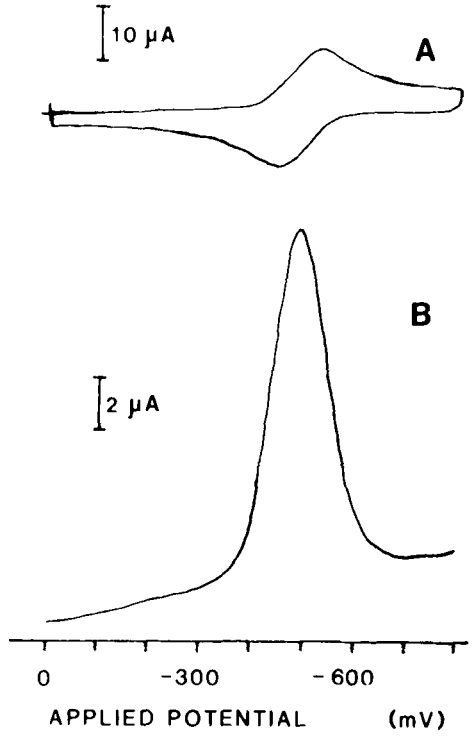


Fig. 2. Cyclic (A) and differential pulse (B) voltammograms of FAD covalently attached to ITO-coated glass, as determined in 0.1*M* Tris buffer, pH 7.4, at 25°C under anaerobic conditions, reference to Ag/AgCl (1*M* KCl). (A) scan rate, 10 mV/s; (B) scan rate, 5 mV/s; 25 mV pulse amplitude, 0.5 s between pulses.

TABLE 1
Cyclic Voltammetry with ITO–Silane–FAD Plates at Different Scan Rates[a]

Scan rate, mV/s	Peak current, μA	Difference in peak potentials, mV[b]
2	0.5	100
5	1.1	100
10	2.3	100
20	5.0	120
50	11.0	150
100	15.0	180

[a]Measured in 0.1*M* Tris buffer, pH 7.4.
[b]Difference betweem reduction and oxidation peaks.

five data points over the scan rate range of 2–50 mV/s. For a redox material attached to the electrode surface, the peak current should be proportional to the scan rate to the first power. This assumes that the surface redox reaction kinetics are rapid compared with the potential sweep rate and the difference between the oxidation and reduction peak potentials is very small and independent of the sweep rate (*20*). From Table 1 it is apparent that neither of these assumptions held true at the higher sweep rates, i.e., 50–100 mV/s, where the electron transfer kinetics could not keep up with the rate of potential sweep (*21*). However, the excellent linearity and the slope of 1.0 at the low sweep rates are good evidence for the presence of attached FAD on the ITO coated plates. ESCA studies have shown that organosilanes form roughly a single monolayer on ITO surfaces (*14*); but the possibility of oxysilane polymerization cannot be ruled out. Thus, the slow FAD kinetics, observed at the higher scan rates, may have been indicative of hindered FAD accessability by silane polymer or simply to slow electron transfer at the electrode surface. Additional studies will be needed to clarify this point.

The presence of attached FAD on the ITO surface also was shown by fluorescence measurements. An emission peak was observed at 512 nm for the ITO–silane–FAD plates. This corresponded very closely to FAD fluorescence in solution, where the emission peak occurred at 514 nm under similar test conditions.

The voltammetry measurements with FAD attached to silanized ITO plates showed little difference in $E^{o'}$ or in the potential at which the reductive peaks occurred for immobilized FAD compared to FAD free in solution. This result is evidence that FAD attachment occurred at the adenine rather than isoalloxazine end of FAD. Since the isoalloxazine ring system is the site of electron transfer in FAD, one would expect a shift in the $E^{o'}$ when FAD is attached to an electrode through a position on the isoalloxazine ring system. However, no such shift in $E^{o'}$ occurred; and moreover very little change would be expected for attachment on the sugar or adenine moieties.

The detailed reaction sequence for attachment of FAD with formaldehyde activation is shown in Fig. 3. The formation of the methylol (—CH_2OH) adduct is certainly the first step. This adduct can lose OH and revert to the immobilized iminium–carbonium intermediate, as shown in Fig. 3. The intermediate in turn is subject to nucleophilic attack by HX-type compounds such as FAD—NH_2. This type of protein amine activation by formaldehyde has been well characterized previously. Caldwell and Gilligan (*22*) isolated ϵ-N,ϵ-*N'*-methylenedilysine (*3*) from the reaction of formaldehyde with wool keratin. Compound **3** results from the simple addition of the lysine amino group to formaldehyde followed by condensation of the N-methylol derivative with another lysinyl side chain, in a reaction similar to that encountered in the present study.

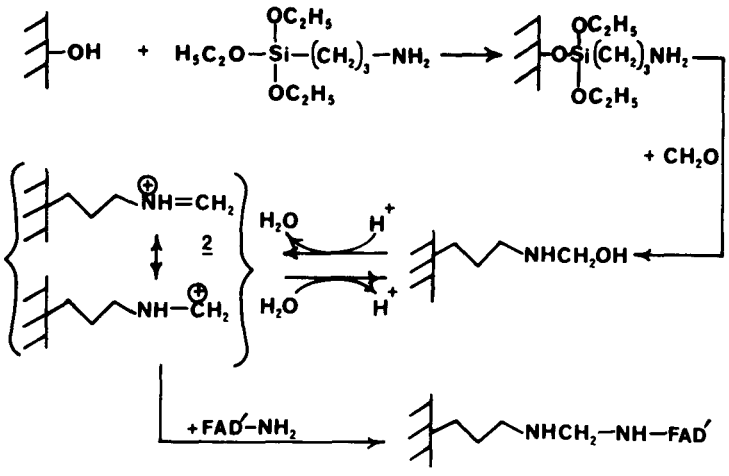
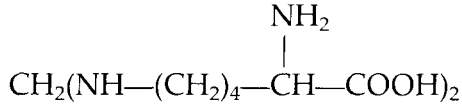


Fig. 3. Reaction sequence for attachment of FAD. FAD′ is defined as FAD except for the adenine amino group; **2** represents the immobilized intermediate.



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Since the immobilized intermediate (**2** in Fig. 3) is not attacked by aliphatic hydroxyl groups (*12*), FAD attachment through the ribityl OH groups can be excluded. However, there is a possibility of nucleophilic attack on intermediate **2** by the N-3 position of the isoalloxazine ring system of FAD (*23*). This possibility is reduced by the presence of a much greater nucleophilic primary amino function in FAD and by the low concentration of immobilized intermediate **2** on the ITO surface. This possibility of N-3 coupling is further precluded by the results of attempts to couple riboflavin, instead of FAD, to the immobilized intermediate **2.** If any coupling at N-3 occurred, it should happen with riboflavin as well as with FAD. When the experiment with riboflavin was carried out, no electrochemically detectable riboflavin was observed on the ITO plates. Thus, all the evidence for the Mannich coupling with FAD points to coupling only through the adenine amino group.

Reaction with Hydroquinone

The presence of the immobilized Mannich type intermediate **2** (*see* Fig. 3) on the ITO plates also was demonstrated by attaching another HX-type compound to the formaldehyde activated ITO-coated aminosilane plates. Hydroquinone was selected as the HX-type compound since (1) the ring hydrogens are active, giving the corresponding ring carbons

nucleophilic character, and (2) the coupled hydroquinone can be readily characterized electrochemically. In a separate paper (*24*), we have demonstrated the use of cyclic and differential pulse voltammetry to characterize *p*-quinone, in this case attached to aminosilane derivatized ITO coated glass plates by 1,4-addition.

In the present paper the attachment of hydroquinone to formaldehyde activated ITO plates was carried out as shown in Fig. 4. The cyclic voltammograms showed an $E^{o'}$ of −380 mV (ref. Ag/AgCl). The differential pulse voltammetry peak occurred at −385 mV. These values compared with a measured $E^{o'}$ of −155 mV for hydroquinone–quinone in solution with the same electrolyte, as determined with an ITO electrode. From a list of published formal potentials for substituted quinones (*25*), it is observed that amine substitution on the quinone ring tends to shift the $E^{o'}$ to more negative potentials. The —NH—CH_2—ring linkage in this study (Fig. 4) and the —NH—ring linkage in another ITO study (*24*) both gave more negative $E^{o'}$ values, in keeping with this observation.

Immobilized FAD Oxidation of NADH

The practical use of electrochemical oxidation of NADH for the analytical determination of substrate or enzyme concentrations or for the regeneration of NAD^+ has been hindered by the roughly 900–1400 mV in overpotential needed to make the reaction proceed (*26,27*). In the present study, a reduction of 180 mV in the NADH overpotential was observed by attaching FAD at the adenine amino position to the ITO electrodes (Fig. 5). With the ITO electrode it was not feasible to sweep the potential to a negative enough value to obtain NAD^+ reduction without also causing reduction of the ITO coating on the electrode. A decrease of 180 mV in the overpotential probably will not be enough to justify practical use of this electrode system for electrochemical analyses of NADH. However, further study of FAD modified ITO electrodes may make it possible to bring about a further reduction in the overpotential for NADH oxidation. The overpotential decrease of 180 mV is similar to the reduction of 195 mV obtained with FAD coupled to glassy carbon at the adenine

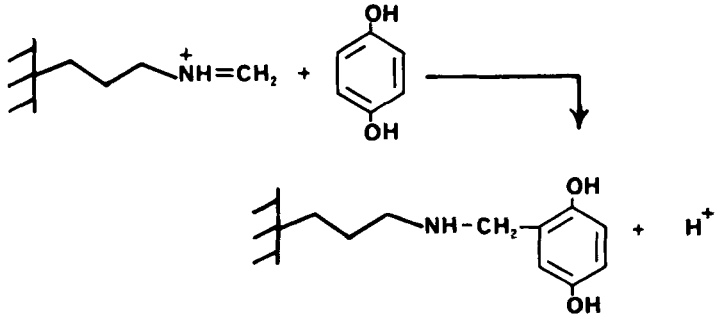

Fig. 4. Reaction of hydroquinone with formaldehyde activated ITO–aminosilane plates.

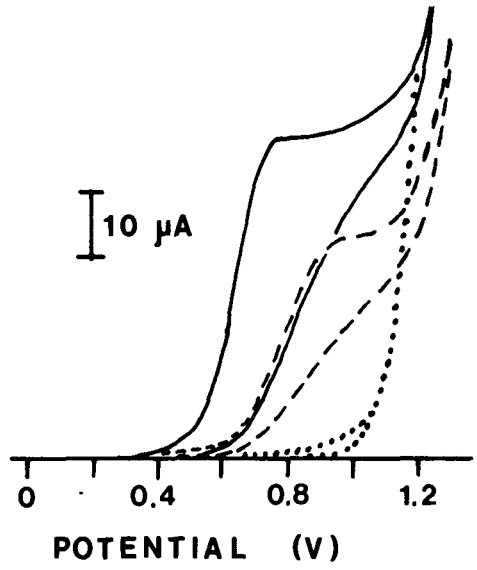

Fig. 5. Voltammograms showing oxidation of NADH in solution at a plain ITO electrode (---) and at an immobilized FAD ITO electrode (—) in 0.1*M* Tris buffer, pH 8.0 (ref. Ag/AgCl). Control electrode (· · ·).

amine or ribityl hydroxy groups (*6*). This indicates that the decrease in overpotential is a function of the FAD and not of the support. This is additional justification for studying further modifications of the FAD molecule for the effect on the ease of NADH oxidation.

Incubation of Immobilized FAD with Apoenzyme of Glucose Oxidase

FAD, attached to the formaldehyde activated aminosilane ITO plates through the FAD adenine amino group, was incubated with glucose oxidase apoenzyme. After washing away loosely attached protein, the plates were tested for enzyme activity. No glucose oxidase activity could be detected. A future study is planned using ^{125}I-labeled apoglucose oxidase to document whether the apoenzyme binds to the attached FAD. These results were consistent with those obtained for FAD attached to glassy carbon through the adenine amino or ribityl OH groups (*6*). However, it does not preclude reconstitution of enzyme activity with a much longer spacer arm to move the FAD further from the electrode surface. Partial reconstitution of glucose oxidase activity did occur with FAD attached to glassy carbon at the isoalloxazine position 8 (*7*) and with FAD adsorbed on spectroscopic graphite (*16*). Additional work is in progress to examine other points on the FAD molecule for attachment to ITO, glassy carbon, or other electron conducting supports for the ability to generate immobilized FAD capable of reconstituting enzymatic activity with apoglucose oxidase or other apoenzymes.

ACKNOWLEDGMENT

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