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IN SITU FTIR THIN-LAYER REFLECTANCE SPECTROSCOPY OF FLAVIN ADENINE DINUCLEOTIDE AT A MERCURY/GOLD ELECTRODE

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Abstract—In this work, *in situ* FTIR thin-layer spectroelectrochemistry has been employed at a flat mercury-based electrode to obtain, uniquely, the spectrum of the reduced form of flavin adenine dinucleotide (FAD) in neutral (pH 7) solutions. The flat mercury surface was achieved by the open-circuit amalgamation of gold and it was found to display the advantageous electrochemical properties, including the high hydrogen overpotential, of mercury. This electrode facilitated the determination of the difference in infrared absorbance resulting from the reduction of a solution of FAD. The spectrum of reduced FAD was recovered from the difference spectrum by adding to it the conventionally determined solution spectrum of the stable oxidized form of the redox couple. The observed shifts in band frequencies for the reduced vs. oxidised forms of FAD are consistent with the prior band assignments by normal coordinate analysis and with the previously proposed sites of the redox chemistry of FAD.

Key words: flavins, thin-layer spectroelectrochemistry, reflectance *ir*, difference spectra, mercury/gold.

INTRODUCTION

Flavin adenine dinucleotide (FAD) is one of two redox-active co-factors of the biologically very important flavoprotein family. The structure of FAD, shown in Fig. 1, consists of the redox-active isoalloxazine system, and the ribityl adenine diphosphate side chain. Depending on the conditions, the isoalloxazine system of FAD can undergo either a one- or two-electron reaction, leading to the semiquinone and hydroquinone products, respectively, during reduction. At pHs between *ca.* 1–2 and 6–7, all three forms of the isoalloxazine moiety remain neutral, *ie* they are neither protonated nor deprotonated[1–3]. At pHs greater than *ca.* 6.5[4, 5], both the one-electron and two-electron reduced products are expected to deprotonate, yielding an anionic form of FAD. In our work, the reduction of FAD is believed to proceed as shown in Fig. 2.

There have been numerous studies conducted regarding the use of *in situ* FTIR thin-layer reflectance spectroelectrochemistry for the elucidation of

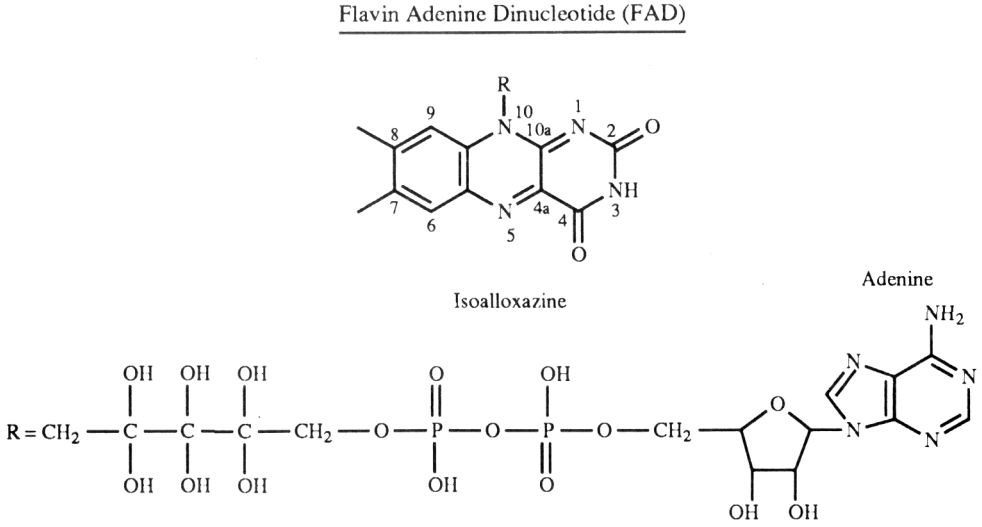
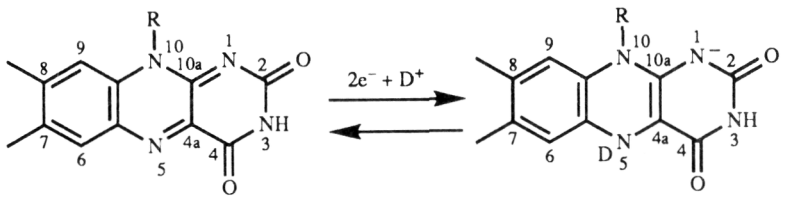


Fig. 1. Structure of flavine adenine dinucleotide (FAD).

FAD Redox Reaction (pH 7):



Isoalloxazine Reduced isoalloxazine

Fig. 2. Electrochemical reduction of FAD in neutral solutions results in addition of two electrons between N_1 and N_5 and D^+ addition to N_5, producing anionic reduced form of FAD.

electrochemical reactions. Such studies generally involve monitoring either the intensity of polarized light reflected from the surface of an electrode as a function of the electrode potential, or the differential intensity associated with the modulation of the polarization of the incident light beam. Such techniques and their applications have been thoroughly reviewed[6]. While the primary objective in most of these types of studies is the characterization of the vibrational spectrum of species attached to an electrode surface, thin-layer reflectance techniques can also be applied to determine the change in infrared absorbance resulting from the oxidation or reduction of species in solution[6–10]. In such studies, polarization of the incident light beam is not necessary and the variation of solution concentrations and/or thin-layer dimensions may be used to advantage[7–10].

In our previous work, we have studied the electrochemical behavior of FAD in dilute solutions in order to observe the behaviour of the adsorbed molecules at a mercury electrode (*hmde*) surface[11, 12]. Three distinct stages of FAD adsorption were observed as a function of time and potential and a model of the FAD orientations was proposed. The model involves an hypothesis that these molecules can adsorb on the Hg surface in either a planar or perpendicular orientation relative to the surface. It is our ultimate desire to test the validity of these hypothesized flavin molecule orientations at electrode surfaces, using FTIR spectroscopy.

In the present work, in order to provide a foundation for subsequent spectroscopic studies to be carried out on adsorbed monolayers of FAD, *in situ* FTIR thin-layer reflectance spectroscopy at a flat mercury-based electrode was employed to obtain both oxidized and reduced solution flavin species spectra. This has permitted the unique determination of the *ir* spectrum of the reduced form of FAD in pH 7 solutions. The reduced form of FAD is normally very unstable, being readily oxidized in all normal environments. Only a few studies of spectroelectrochemistry at mercury electrodes have been reported, first on mercury pool electrodes[13, 14], and more recently on a mercury-coated gold electrode[15]. These prior results and particularly those of the present study suggest that many other redox active species with relatively negative redox potentials requiring the high hydrogen overpotential of Hg can now be studied by FTIR reflection spectroscopy. In addition, the present results are promising in terms of the possibility of testing the previously hypothesized orientations of adsorbed FAD[11, 12] on mercury electrodes using FTIR reflectance spectroscopy in the near future.

EXPERIMENTAL

(a) *Electrodes and cells*

The working electrode used for the spectroelectrochemical experiments consisted of a mercury-coated gold disc (7 mm diameter, Aldrich, 99.99% pure) welded to a brass rod which was press-fitted into a Teflon holder. Prior to mercury coating, the gold surface was polished to a mirror finish with successive grades of diamond paste (down to 0.25 μm). A shiny and stable mercury coating was achieved by immersion of the gold electrode into a pool of mercury (triply distilled, Bethlehem Apparatus Co.) for approximately 3 min. Excess mercury was removed by suction, leaving a surface which had the physical properties of a solid surface, but which behaves electrochemically like mercury. The counter electrode was a Pt wire and the reference electrode an *sce*. The cell window of the spectroelectrochemical cell was composed of CaF_2. This cell has been described in detail in previous publications[8–10].

(b) *Equipment*

Spectroelectrochemistry was carried out using a PAR 174A polarographic analyzer for potential control. The spectra were obtained with a Nicolet 8000 Fourier transform spectrometer employing a MCT narrow-range detector. All spectra were recorded with 4 cm^{-1} resolution.

(c) *General*

The spectroelectrochemical experiments were carried out in buffered (phosphate) near-neutral D_2O solutions. FAD was obtained as its sodium salt from Sigma Chemicals Co. The flavin solution concentration ranged from 3 to 5 mM. All solutions were deoxygenated with nitrogen prior to obtaining the spectra.

The infrared difference spectrum is reported as the difference between absorbance of the thin-layer solution at a potential of − 350 mV, where the FAD is in the oxidized form, and − 550 mV, where reduction of the FAD is complete. The methodology, instrumentation and optical configuration were as described in previous publications[8, 10].

RESULTS AND DISCUSSION

(a) *General electrochemical behaviour of FAD at* Hg-*coated* Au *electrodes*

Figure 3 shows a typical cyclic voltammogram obtained for a solution of FAD of 0.5 mM concentration at pH 7 using a Hg/Au electrode. In this case, the Hg/Au electrode was pulled well back from the cell window by about 2 cm. The observed diffusion-controlled response is typical for FAD at Hg electrodes[11], revealing that the reaction is not fully reversible at the sweep rates examined. It is noteworthy that FAD is in its fully oxidized state in solution, particularly in the presence of oxygen. The similarity of the cyclic voltammograms obtained using a pure mercury electrode (11) and the Hg/Au electrode in Fig. 3 clearly demonstrates that the Hg/Au electrode does not introduce any artifacts into the FAD electrochemistry, even revealing the high overpotential for hydrogen evolution expected of mercury.

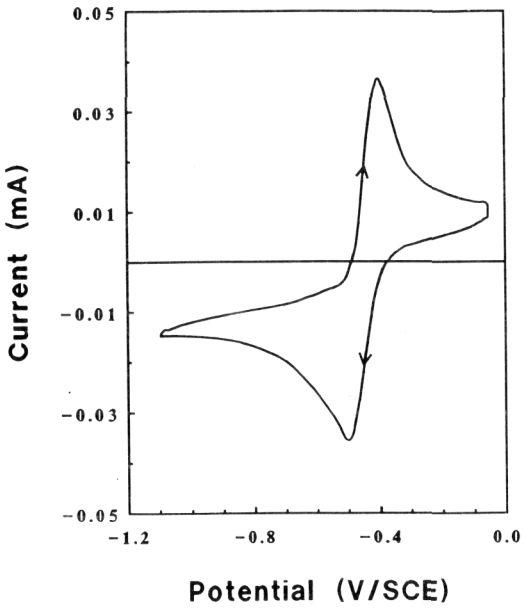


Fig. 3. Typical cyclic voltammogram ($s = 100\,mV\,s^{-1}$) in pH 7 buffered D_2O solution containing 5 mM FAD.

(b) *Spectroelectrochemical study of FAD in solution*

Figure 4 illustrates the infrared absorbance spectrum of FAD in the region 1350–1800 cm^{-1}. This was observed for a 5 mM solution in D_2O containing phosphate buffer (pH 7) in a 0.02 mm pathlength cell. Abe and Kyogoku have carried out a normal

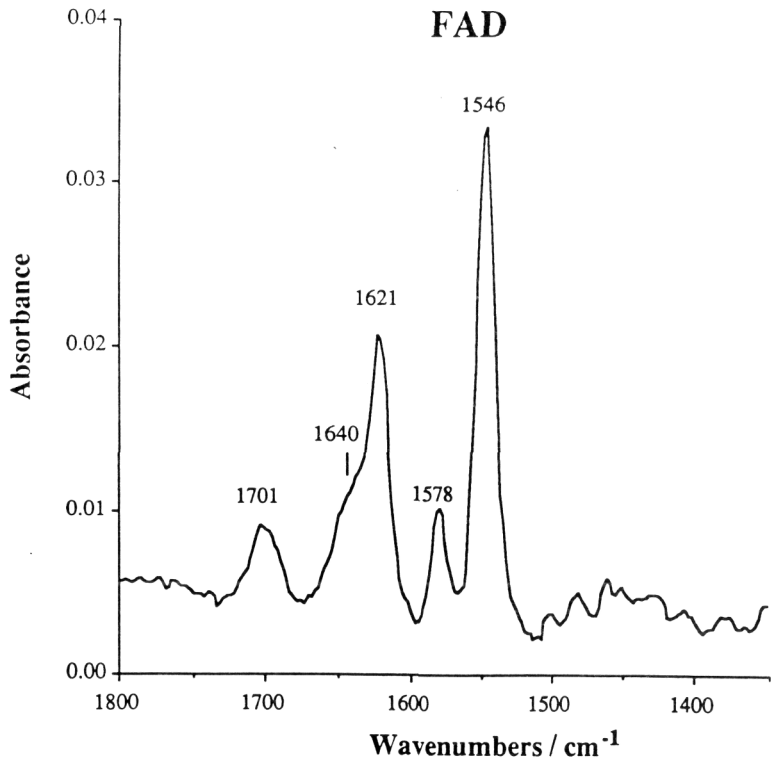


Fig. 4. *ir* Absorbance spectrum of 5 mM FAD solution (pH 7, D_2O) in 0.02 mm pathlength cell. FAD is in fully oxidized form under these conditions.

coordinate analysis of the isoalloxazine ring system in lumiflavin, which carries only a CH_3 substituent at the N_{10} position[16]. By comparison with their work, the infrared bands observed for FAD have been assigned as shown in Table 1. The bands at 1701 cm^{-1} and the shoulder at 1640 cm^{-1} correspond to ν_4 and ν_5, and arise primarily from C=O stretching vibrations. The band at 1578 cm^{-1} corresponds to ν_8 and is primarily associated with N_1C_{10a} and $N_{10}C_{10a}$ stretching while the strong band at 1546 cm^{-1} arises from N_1C_{10a} and $C_{4a}N_5$ stretching. The absorbance at 1621 cm^{-1} is most likely associated with the adenine ring system in FAD. This is inferred from the observation of only very weak absorbance at this position in flavin mononucleotide (FMN). FMN is similar to FAD but is missing the adenine group, ribosyl ring and one of the phosphate groups.

Figure 5 shows a cyclic voltammogram obtained for a thin layer of 5 mM FAD solution of pH 7 prepared in D_2O. The thin-layer thickness was *ca.* 6 μm, as estimated from the charge required to completely reduce FAD. The thin-layer dimension was readjusted once the cell was mounted in the FTIR spectrometer to achieve optimum signal to noise ratio for the

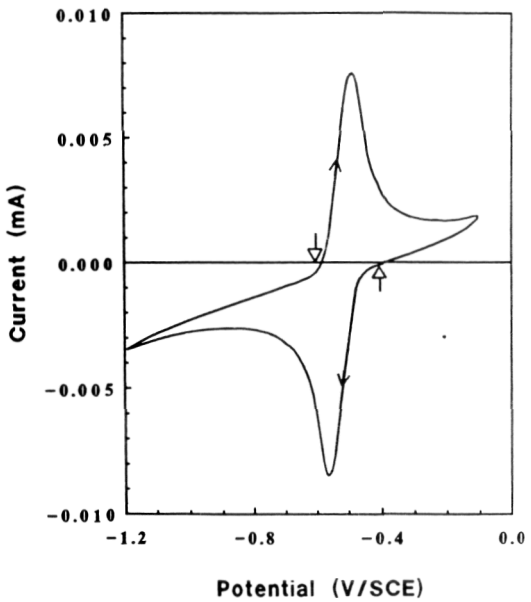


Fig. 5. Typical thin-layer cyclic voltammogram ($s = 5\,mV\,s^{-1}$) in pH 7 buffered D_2O solution containing 5 mM FAD. Arrows indicate potentials at which spectra were collected.

infrared measurements. Figure 6 shows the difference in absorbance observed in the thin layer reflectance spectroelectrochemical cell when the potential was stepped from −350 to −550 mV. The same solution as was used to record the infrared spectrum of Fig. 4 was used. Under these conditions, the isoalloxazine ring system undergoes reduction by two electrons and one deuteron to produce $FADD^-$, as shown in Fig. 2. This difference spectrum is marked by the absence of noise and the clarity of the spectral features, indicative of a well-behaved spectroelectrochemical system.

The most intense feature in the difference spectrum of Fig. 6 is a strong negative absorbance at 1545 cm^{-1}. This corresponds to the disappearance of the 1546 cm^{-1} absorbance band, observed in Fig. 4 for unreduced FAD. Since the optical pathlength associated with Fig. 4 was known to be 20 μm, comparison of the intensity of the 1546 cm^{-1} absorbances in the spectra of Figs 4 and 6 facilitated calculation of the optical pathlength associated with the difference spectrum of Fig. 6. This was found to be 54 μm. This corresponds to a thin-layer thickness of 19 μm for the 45° angle of incidence employed[10].

One of the goals of this work was to determine, for the first time, the *ir* spectrum of the reduced form of FAD. Figure 7 presents the infrared absorbance of the reduced species, $FADD^-$. This was obtained by adding the absorbance spectrum of FAD back into the difference spectrum of Fig. 6. The larger absorbance scale in Fig. 7 for $FADD^-$, as compared to that in Fig. 4 for FAD, results from the longer optical pathlength employed in the former case and does not imply enhanced molar absorptivity for bands of $FADD^-$, as compared to those of FAD. The suggested assignments for the infrared bands at $FADD^-$ are summarized in Table 1.

The increased electron density in the isoalloxazine ring system resulting from reduction is anticipated to result in a decreased polarity of the C=O bonds. This, in turn, will cause a shift of their vibrational frequencies to lower wavenumbers. Thus, it seems likely that the C=O vibrational modes giving rise to the 1701 and 1640 cm^{-1} absorbance bands in FAD are responsible for the absorbances at 1678 and 1600 cm^{-1} seen in the infrared spectrum of the reduced species, $FADD^-$.

In comparing the structures of FAD and $FADD^-$, it is apparent that bonds N_1C_{10a} and N_5C_{4a} possess primarily double bond character in the oxidized form, but primarily single bond character in the reduced species. The absorbance at 1546 cm^{-1} in the spectrum of FAD, which reflects stretching of these

Table 1. Infrared band frequencies and assignments in the 1800–1350 cm^{-1} range observed for FAD and $FADD^-$ in D_2O solution

Band	Assignment	Observed frequency (cm^{-1})	
		FAD	$FADD^-$
ν_4	$\nu(C_4O)$, $\nu(C_2O)$	1701	1678
ν_5	$\nu(C_4O)$, $\nu(C_2O)$	1640	1600
	Adenine	1621	1624
ν_8	$\nu(N_1C_{10a})$, $\nu(N_{10}C_{10a})$	1578	1513
ν_9	$\nu(N_1C_{10a})$, $\nu(N_5C_{4a})$	1546	1400

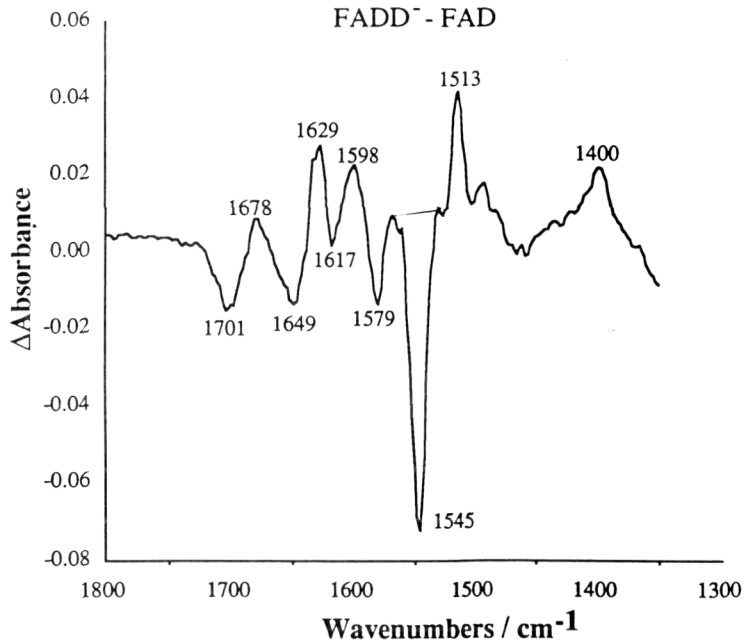


Fig. 6. Difference in spectra obtained for reduced (− 550 mV) and oxidized (− 350 mV) forms of FAD.

two bonds, would thus be expected to undergo a large shift to lower energy. This vibrational mode is thus likely to account for the 1400 cm^{-1} absorbance in the spectrum of the reduced species. The 1578 cm^{-1} band in FAD, arising from N_1C_{10a} and $N_{10}C_{10a}$ stretching, would similarly be expected to shift to lower frequency, although not to the same degree as the 1546 cm^{-1} band. This mode can then be assigned to the 1513 cm^{-1} absorbance apparent in the spectrum of $FADD^-$.

The 1621 cm^{-1} band in the spectrum of FAD apparently undergoes little, if any, perturbation on reduction. This observation is clearly consistent with assignment of this band to the adenine ring system,

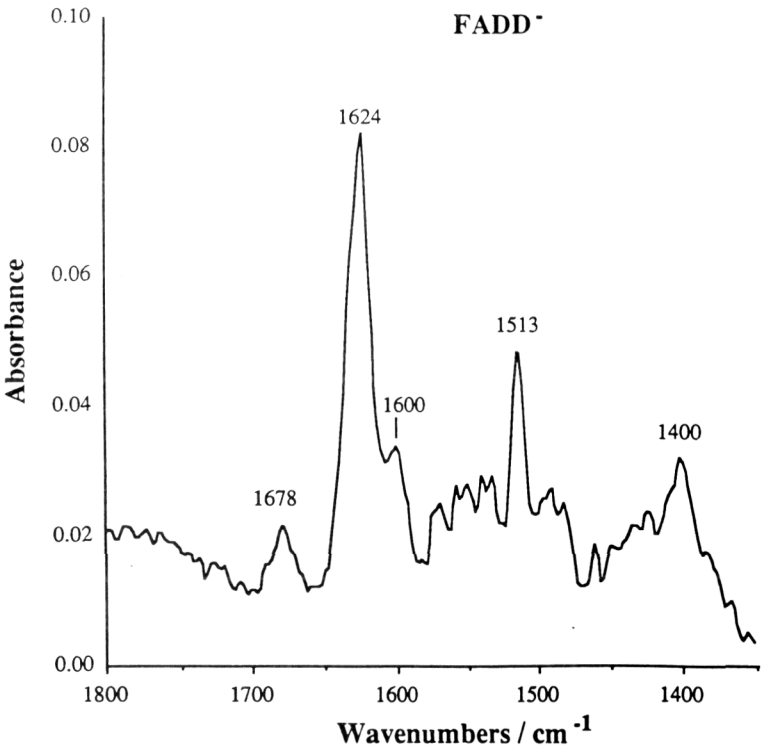


Fig. 7. Spectrum of reduced FAD obtained from addition of difference spectrum (Fig. 6) and spectrum of oxidized FAD (Fig. 4).

since this portion of the molecule is expected to be unaffected by reduction of the isoalloxazine system in FAD. Although normal coordinate analysis of large molecules is sometimes open to question, the fact that the changes in the spectral features of the oxidized vs the reduced forms of FAD agree with those predicted from the normal mode assignments[28] provides confirmation of the correctness of these assignments. Also, this good agreement, which involves substantial changes in the N(1) and N(5) environments, supports the literature suggestions[5, 29–31] that these are the sites of the redox chemistry of FAD.

The Hg-based electrode prepared in this work has been found to be very suitable for reflectance *ir* studies and will permit future work with redox systems which require the high overpotential of mercury, and hopefully prove to possess the best surface for the study of the orientations of monolayers of adsorbed FAD.

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