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Journal of Electroanalytical Chemistry 423 (1997) 13–21

JOURNAL OF ELECTROANALYTICAL CHEMISTRY

An electrochemical study of the photolysis of adsorbed flavins [1]

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Received 8 January 1996; revised 16 July 1996

Abstract

The adsorption behaviour of four members of the flavin family was examined in pH 7 solutions at Hg electrodes, both in the dark and when exposed to UV–visible light. In the absence of illumination, all flavins, which share the redox-active isoalloxazine ring system, adsorb strongly, initially in a parallel orientation and then undergo reversible, potential-driven orientational changes, from parallel to perpendicular, at higher surface coverages. Photolysis of flavin solutions (but not lumiflavin) yields two photoproducts, as detected electrochemically by their adsorption behaviour, and is supported by solution spectral analyses. The principal photoproduct is lumichrome, formed through an intramolecular photodealkylation reaction; it adsorbs strongly at Hg and forms a compact monolayer which undergoes a reversible redox reaction at ca. 100 mV negative of the flavin potential. A second product, which oxidizes at ca. 150 mV positive of the flavins and is detected electrochemically only when photolysis is carried out in the presence of oxygen, may result from an intramolecular photoaddition reaction involving the bridging of a hydroxyl group on the substituted chain to the isoalloxazine ring system.

Keywords: Flavins; Adsorption; Photolysis; Electrochemistry

1. Introduction

The flavoproteins play an important biological role in the electron transfer reactions of living systems, primarily because of the presence of the redox-active cofactor, flavin adenine dinucleotide (FAD). Other members of the flavin family include flavin mononucleotide (FMN), riboflavin (RF) and lumiflavin (LF) [1,2]. All flavins have in common the isoalloxazine ring system, which is their redox-active component and which reacts by the addition of either one or two electrons (along with protons) to the two double bonds between N-1 and N-5 (Fig. 1). The nature of the R group attached at the N-10 site is what differentiates the members of the flavin family (Fig. 1).

Flavins, in particular RF, have been studied electrochemically, primarily at mercury electrodes, in the past. The earlier research involved polarographic studies [3,4], while later work included a.c. polarography and chronopotentiometric methods [5–9]. Although reported to be strongly adsorbed on Hg, in both its reduced and oxidized states in acidic solutions [5,6,10], the reduction of RF from solution occurs in a facile reversible two electron transfer reaction [9]. More recently [11], it was shown that both the oxidized and reduced forms of FMN adsorb at Hg in buffered pH 4.9 and 6.9 solutions [12], and that FAD is also strongly adsorbed at a pH of 6.9 [13].

An area of current interest involves the attachment of flavins to electrode surfaces, e.g. FAD, in order to mediate glucose oxidation and serve as a glucose sensor [14–23], as well as for the sensing of biomolecules such as cytochromes and methaemoglobin. One approach to surface immobilization has been to attach flavins through mercaptan [20,21] and thiourea [22] linkages to noble metal substrates. Flavins have also been coated on Au with the use of the Langmuir–Blodgett technique [23].

In our prior work [24,25], it has been shown that FAD adsorbs very strongly in two different orientations on a mercury electrode surface in neutral solutions. At dilute coverages, it has been suggested that both the isoalloxazine and adenine moieties are adsorbed parallel to the electrode surface at all potentials, while at higher surface coverages, FAD can reorient to a perpendicular configuration [24,25], particularly at potentials near the pzc. In acidic solutions [26], evidence for a third tightly packed orientation has been obtained, in which the adenine group, now protonated, is no longer on the electrode surface, resulting in

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[1] Dedicated to Petr Zuman on the occasion of his retirement from Clarkson University.

PII S0022-0728(96)04881-4

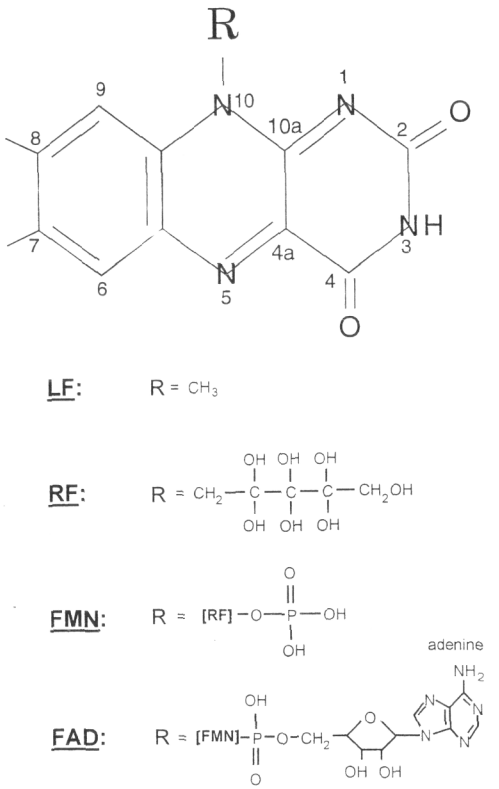

Fig. 1. Oxidized form of the isoalloxazine ring system and members of the flavin family, as defined by the nature of the R group attached to N-10.

improved lateral interactions and tighter packing of adjacent isoalloxazine ring systems. The adsorbed FAD monolayer is believed to reorient, concertedly, between parallel and perpendicular, as the potential is changed in all solutions. A detailed study of the adsorption behaviour of lumiflavin [27] and of the comparative electrochemical behaviour of the flavin family [28] over a wide pH range is currently under preparation.

The objectives of the present work have been to survey the adsorptive behaviour of the four flavins, i.e. FAD, FMN, RF and LF, at Hg electrodes, in pH 7 solutions, and primarily to determine the effect of visible light on their electrochemical responses. It was hoped that further insight could be gained from these photolysis experiments into the adsorptive and orientational behaviour of the flavins on the mercury electrode surface. In the course of this work, it was found that two photoproducts are formed in the case of adsorbed FAD, FMN and RF. Therefore, a further objective of this research became to identify these photoproducts and also to understand their behaviour at Hg electrode surfaces.

2. Experimental

2.1. Electrochemical equipment

Either an EG&G PARC 173 or a Hokuto Denko Ltd. HA301 potentiostat was used in conjunction with an EG&G PARC 175 programmer for the CV experiments. All cyclic voltammetric data were plotted on an SE-780 BBC or a HP7045A X/Y recorder.

2.2. Cell and solutions

The CV experiments were carried out in the standard cell supplied with the EG&G 303 apparatus. The cell was wrapped in aluminium foil to prevent exposure in light sensitive experiments. The working electrode (WE) was the hanging mercury drop electrode (medium setting, 0.017 cm^2), while the counter electrode was a Pt wire. An Ag|AgCl|4 mol l^{-1} KCl electrode was used as the experimental reference electrode (RE), which was calibrated against a standard calomel electrode (SCE) on a regular basis. All potentials reported in this paper are given versus the SCE.

All of the experiments were carried out using a pH 7 phosphate buffer solution. The flavin compounds and lumichrome were supplied by Sigma Chemicals; both the FMN and FAD were obtained in the form of their sodium salts. The concentration of the flavin solutions ranged between ca. 5 and 10 μM for the photolysis experiments, and 0.5 and 3 μM in the electrochemical cell. All chemicals were of reagent grade and water was triply distilled. The electrochemical and photolysis cell solutions could be deaerated by bubbling nitrogen either through or above the solutions during the experiments. All experiments were carried out at room temperature, i.e. ca. 22°C.

2.3. Photolysis experiments

Photolysis was carried out using a 150 W (24 V) tungsten–halogen lamp. The flavin solution (about 10–25 μM) under photolysis was contained in a 250 ml volumetric flask, either in the aerated or deaerated (nitrogen bubbling through the solution) condition, and placed in a lightproof enclosure. 2 and 5 ml aliquots were withdrawn by pipet from the stock solution at periodic intervals for the electrochemical and spectral analyses respectively. UV–visible spectra were collected using a Shimadzu UV240 model spectrophotometer, with quartz cuvettes and pH 7 buffer as the standard solution.

3. Results and discussion

3.1. General electrochemical behaviour of flavins at Hg (dark experiments)

Fig. 2(a) shows the series of cyclic voltammograms which are observed when a fresh Hg drop surface is exposed to a quiescent ca. 2 μmol l^{-1} FAD solution, buffered to pH 7. Although these experiments were carried out with exposure to laboratory lighting, the time of experimentation was short and hence light can be considered to

have no effect on the observed behaviour. As the FAD concentration is so dilute and the sweep rate relatively high ($100\,mV\,s^{-1}$), only the oxidation/reduction response of adsorbed FAD is seen [24]. With continuous cycling, the FAD redox peaks increase in size at a diffusion controlled rate [24,26]. An increase in the concentration of FAD in solution or the presence of any solution agitation would have caused the FAD peak to reach its full size in a much shorter time period.

In the first four cycles shown in Fig. 2(a), the anodic and cathodic peaks are symmetrical in shape and the small capacitive peaks at ca. −0.15, −0.3, −0.4 (as a shoulder on the anodic peak) and −0.85 V, reflecting the potential-dependent reorientation of FAD on the Hg surface [24], do

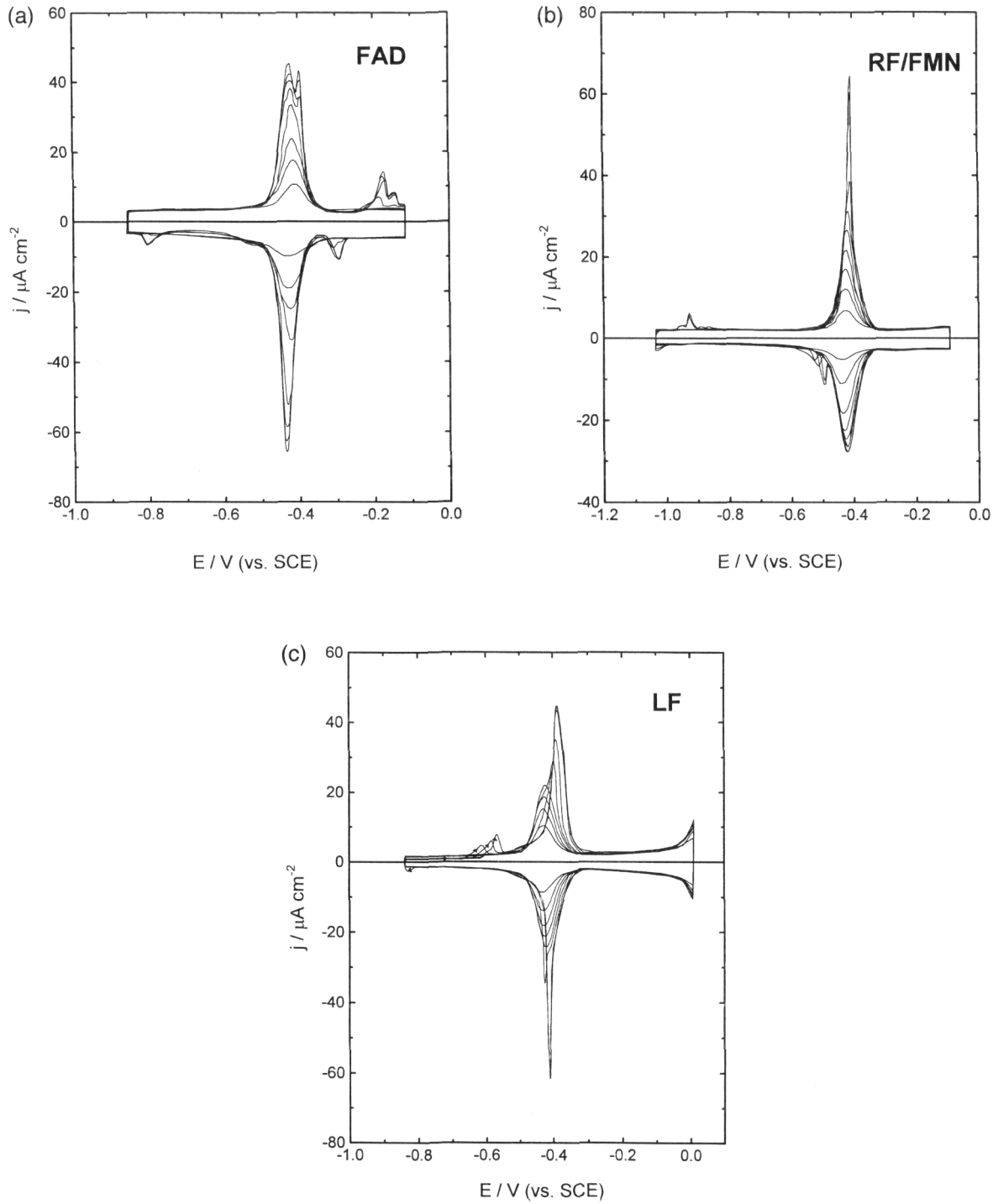

Fig. 2. CV response of a freshly exposed Hg drop electrode to deaerated 2 μM flavin solutions (pH 7), $s = 100\,mV\,s^{-1}$, no stirring. Peaks increase with time as flavin adsorbs to a maximum of one monolayer.(a) FAD; (b) FMN and RF; (c) LF.

Table 1
Surface coverage of adsorbed flavins at Hg (3 μM flavin pH 7 solutions)

Flavin	Experimental charge density/μC cm^{-1}		Predicted area per molecule/Å^2	
	Stage I	Stage II	Stage I	Stage II
LF	17	25	195	130
FMN	13	20	255	165
RF	15	20	220	165
FAD	9	14	370	240

not appear yet. The completion of this stage of adsorption, labelled previously as Stage I [24], involves ca. 9 μC cm^{-2} and has been suggested to reflect the adsorption of both the isoalloxazine moiety and the adenine end group in an orientation parallel to the Hg surface [24,26]. This charge density is equivalent to an area per FAD molecule of ca. 370 Å^2 in this configuration (Table 1), clearly a fairly large occupied area, consistent with the proposed parallel adsorption scheme and steric contribution of the long chain joining the adenine and isoalloxazine groups (Fig. 1).

With further cycling of potential (Fig. 2(a)), more FAD adsorbs, up to a saturation charge density of ca. 14 μC cm^{-2} (240 Å^2 per FAD), consistent with the more compact structure in which both the isoalloxazine and adenine groups are adsorbed in a vertical orientation (Stage II) [24]. In Stage II of adsorption, the redox peaks are narrower in shape and all of the capacitive features referred to above are well developed, increasing in size with increasing FAD surface coverage.

Based also on a.c. voltammetry results, it was reported earlier [24,26] that the small capacitive features in Fig. 2(a), seen for the tightly packed monolayer, can be interpreted as follows. At potentials positive of ca. −0.25 V, the isoalloxazine moiety is parallel to the surface and the adenine is probably no longer in contact with the Hg substrate. In the negative scan, the capacitive peak at ca. −0.3 V represents the reorientation of the FAD molecule such that both the isoalloxazine, and presumably the adenine, are now in a more tightly packed perpendicular orientation, while at potentials negative of ca. −0.8 V, the molecule reorients to parallel once again. In the positive sweep, adsorbed FAD retains this orientation until oxidation is almost complete, at which point it reorients vertically once again in the capacitive shoulder on the positive side of the anodic peak, only to reorient once again to the parallel configuration at the positive end of the scan. This process is depicted schematically for FAD in Fig. 3(a), using highly simplified rectangular symbols to represent the isoalloxazine and adenine groups, expected to be the surface-active components of the FAD molecule [24,26]. Note that this figure does not reflect the anomalous stability of FAD in its parallel orientation once it is reduced, i.e. from potentials negative of −0.8 V and then to positive of ca. −0.4 V, or even the hysteresis of ca. 200 mV between the reorientation peaks at the positive end of the potential scale.

Analogous sets of CVs for RF and FMN (Fig. 2(b)) and LF (Fig. 2(c)) were obtained under essentially identical conditions to those used for FAD. FMN and RF are very similar in behaviour, perhaps consistent with their similar structures (Fig. 1), and therefore their CVs, which are almost indistinguishable under these conditions, are shown superimposed in Fig. 2(b). The three sets of CVs in Fig. 2 clearly have numerous common features, the most important being that the redox potential of the peaks is centred near −0.43 V in all cases. This is very close to the reported potential for the solution redox reaction of flavins, i.e. in the range −0.45 to −0.46 V [24,26,29–31]. Also, all of the flavins exhibit Stage I of adsorption, where symmetrical anodic and cathodic peaks are seen, followed by a transformation to a second configuration, i.e. Stage II, as seen by the narrowing of one or both of the redox peaks, the appearance of the small capacitance peaks (indicative of adsorbate reorientation [24,26]), and a marked decrease in the double layer capacitance. In the case of LF, Fig. 2(c) also shows that extension of the potential negative of −0.8 V yields a further decrease in capacitance, which increases again at potentials positive of the more

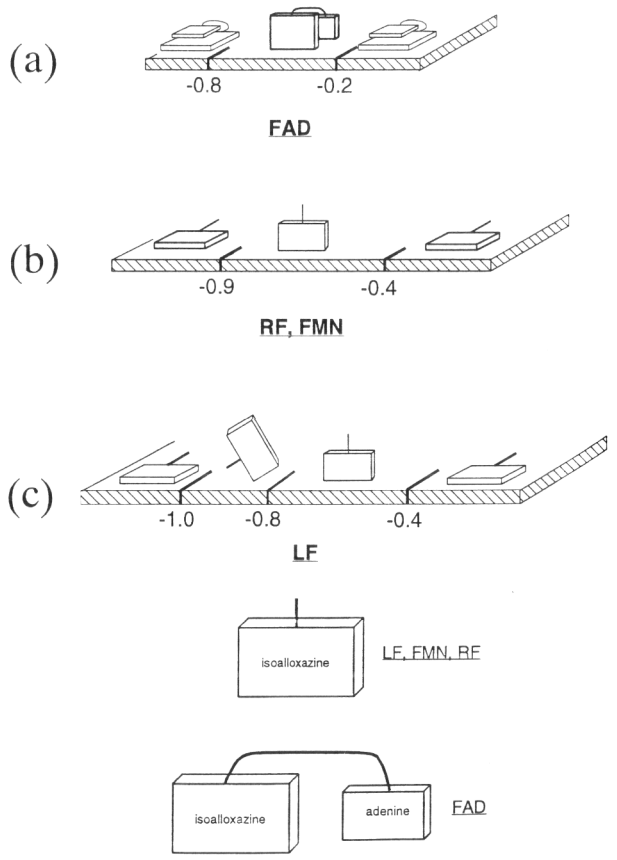

Fig. 3. Proposed orientations of flavins at saturation coverage as a function of potential versus SCE (see Fig. 2). Large rectangles represent the isoalloxazine ring system, small rectangles the adenine group. (a) FAD; (b) FMN and RF; (c) LF.

positive capacitance peak at −0.65 to −0.6 V. Also, time spent in the −0.8 to −1.0 V range results in a higher surface coverage of LF than observed during normal potential cycling procedures [27]. Therefore, a more condensed form of the film is proposed to develop at potentials between ca. −0.8 and −1.0 V [27]. Another set of capacitance peaks, analogous to those seen for RF and FMN in Fig. 2(b), are also seen for LF at −1.0 to −1.1 V [27].

A schematic representation of the reorientation processes which are believed to occur in the case of RF and FMN are shown in Fig. 2(b) and for LF in Fig. 2(c). LF, being the smallest of the flavins, is the most labile, as determined by its more complex adsorption behaviour [27]. One of the tests of the proposed parallel and perpendicular orientations of adsorbed flavins is the charge density passed to maximum coverage at the completion of Stages I and II in Fig. 2. These values are given in Table 1, along with the calculated areas per adsorbed molecule. These areas can be seen to be quite reasonable, based on the estimated areas of the isoalloxazine (ca. 100 $Å^2$) and adenine (ca. 60 $Å^2$) groups and the hypothesized orientations shown in Fig. 3. It can also be seen that the relative magnitudes of the charge densities for the different flavins are consistent with LF being the smallest and FAD the largest (Fig. 1).

3.2. The effect of light on adsorbed flavin response

The effect of light on flavins in solution is relatively well known [32–35]. In the present work, this could be detected easily, particularly when working with RF and FMN, in the change of colour of both the cell and stock solutions from bright yellow to nearly colourless with time of exposure, even to laboratory lighting. Also, pronounced changes are observed in the CV response of RF and FMN with time of experimentation under these conditions. A typical example of the CV response of a fresh Hg drop electrode as RF adsorbs from a deaerated 2 μmol l^{-1} solution after the cell solution was exposed for some time to laboratory lighting is shown in Fig. 4. It is clear that several new features have appeared, most notably the redox peak centred at ca. −0.53 V, but also the anodic peak seen at −0.3 V, shown only for the 28th cycle of potential. It is of interest that changes of this kind take place much more slowly in the case of FAD solutions, especially in acidic and neutral conditions, and do not occur at all for LF. In order to study the effect of illumination on flavin adsorption more rigorously, controlled photolysis experiments were carried out, employing a tungsten–halogen lamp. This provided a substantially more intense and controllable light source than laboratory lighting, and also allowed most experiments to be completed in several hours, compared with several days.

Fig. 5 shows an example of the final steady state CVs seen at a fresh Hg electrode after the photolysis of an RF solution for controlled periods of time using the tungsten source. These experiments were carried out by continuously photolyzing a 10 μmol l^{-1} RF stock solution, periodically withdrawing a ca. 2 ml aliquot of this solution into a clean electrochemical cell, diluting to 10 ml with pH 7 phosphate buffer and then running the adsorption CVs. Only the final steady state CV for each solution is shown in Fig. 5. A second ca. 5 ml aliquot was withdrawn in

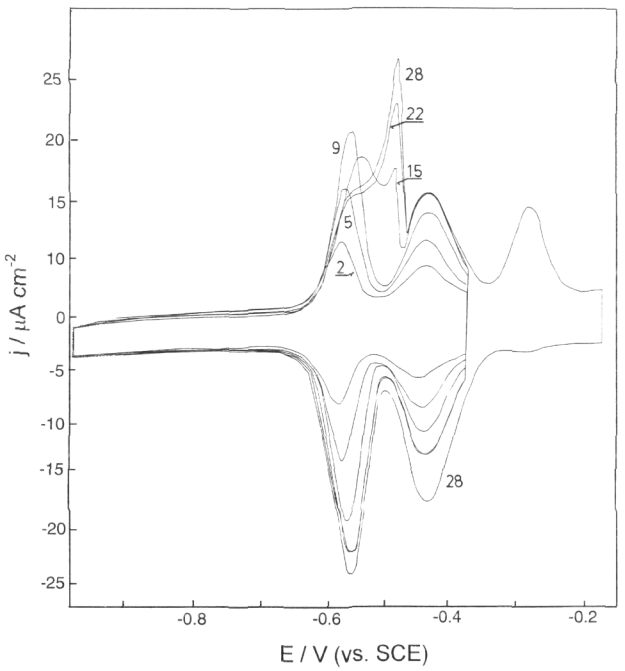

Fig. 4. CV response observed at a fresh Hg surface during adsorption of RF from a partially photolyzed pH 7, 2 μM RF solution, $s = 100\,mV\,s^{-1}$. Cycle numbers are shown in the figure.

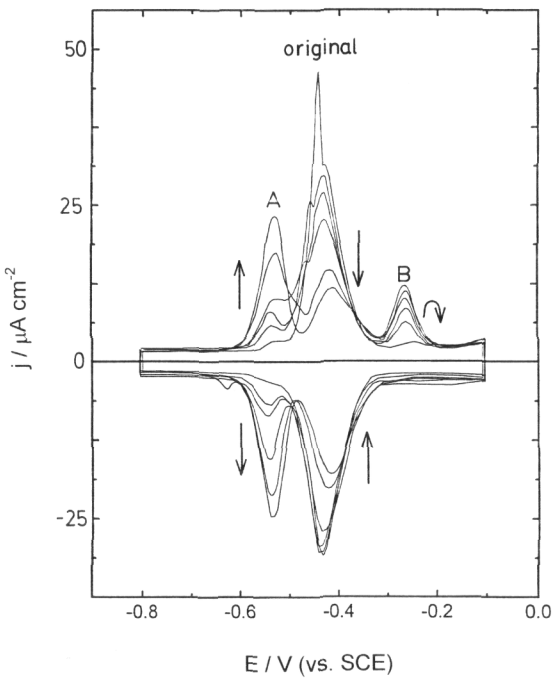

Fig. 5. CV response of a fresh Hg electrode to 1.6 μM RF solution (pH 7) after various times of photolysis. Times of exposure to tungsten light source are 0, 20, 30, 50, 80 and 120 min.

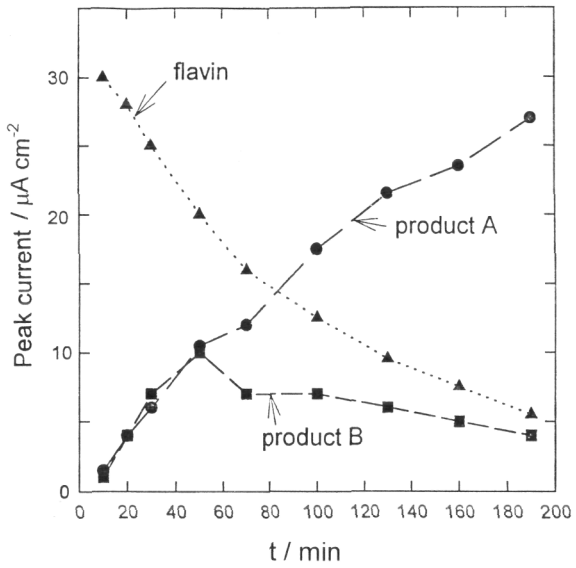

Fig. 6. Magnitude of CV peaks from Fig. 5 as a function of time of photolysis. 'Flavin' refers to original RF redox peaks at −0.43 V, product A is centred at −0.53 V and product B is seen at −0.25 V.

parallel for subsequent spectral analysis at each time interval. The first of the CVs shown in Fig. 5, i.e. for a solution which had not been photolyzed, is similar to the final scan shown in Fig. 2(b) for RF, as expected. Fig. 5 shows that, after several hours of illumination, the original RF redox peak at ca. −0.43 V has become quite small, and that the two new features, peaks A and B, also seen in Fig. 4, have again developed. Peak A, centred at ca. −0.53 V, clearly increases in size as the original RF peak decreases, while the anodic peak labelled B in Fig. 5 at ca. −0.25 to −0.30 V increases and then decreases somewhat after further photolysis.

Fig. 6 shows a plot of the magnitude of these three peaks as a function of time of photolysis for the case of RF. A very similar time dependence was seen for FMN solutions. In the case of FAD, to achieve a diminishment of the original FAD redox peak by 50%, illumination for 30 to 40 times longer than this was required, under otherwise identical conditions. As stated above, LF solutions were completely impervious to any changes as a result of photolysis.

The UV–visible spectra of the 5 ml aliquots of the photolyzed stock solution, gathered in parallel with the electrochemically studied samples, are shown in Fig. 7. Pronounced spectral changes are seen at ca. 450, 370/380 and 280 nm, as the original RF bands decrease in size. An increase in absorbance is seen at 220, 260 and 350/360 nm, consistent with the build-up in photoproduct A. Interestingly, a small band at ca. 400 nm initially increases and then decreases somewhat, perhaps reflecting the electrochemical behaviour of photoproduct B.

Other interesting observations concerning the photolysis process include the fact that photoproduct B is generated only when photolysis is carried out in the presence of oxygen. This was tested in several experiments with FMN, RF and FAD by removing oxygen by bubbling nitrogen either through or above the stock flavin solutions throughout the time of illumination. The CV peak at −0.25 V was then not observed. Also under anaerobic conditions, the photogeneration of product A is significantly faster, i.e. by a factor of 2 or 3 compared with the case of non-deaerated conditions.

It is also notable that while product B can be oxidized only at a potential positive of the flavin redox potential, perhaps reflecting some kinetic limitations of this reaction, it reduces electrochemically at the flavin potential. This is seen clearly in Fig. 4 for the partially photolyzed 2 μM RF solution, where extension of the potential positively over peak B (at −0.3 V) results in an enhancement primarily of the RF reduction peak at −0.45 V in the negative scan. Had the potential been extended up to −0.2 V in all cycles, the anodic peak B would have been seen to increase continuously with time. The oxidation product of B is therefore not the original flavin as, if this were the case, peak B would not have increased with time of cycling in this partially photolyzed solution. These results indicate that photoproduct B is likely to be a unique product, which probably retains the flavin isoalloxazine ring structure, such that it is at least reducible at the expected flavin potential.

Fig. 4 also shows that, as the amount of the original RF in solution diminishes, and the peak for product A increases, the RF redox peak becomes broader in shape, more similar to that of the Stage I peak seen at lower surface coverages (Fig. 2(b)), even while the total surface coverage (of RF plus photoproducts A and B) is still rather high. The fact that RF and the photoproducts appear to be behaving independently, i.e. with the flavin redox peaks

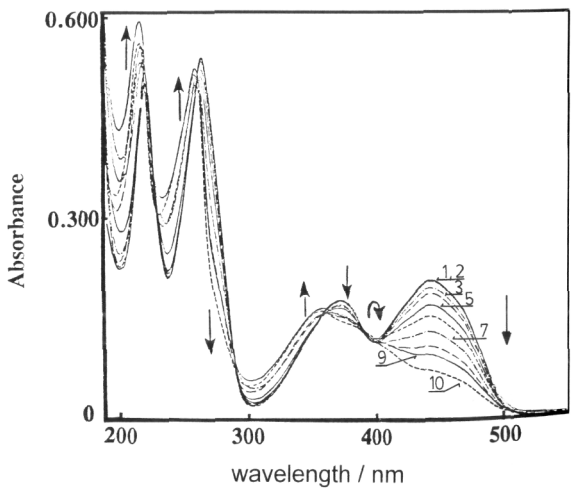

Fig. 7. UV–visible spectra as a function of time of photolysis of 10 μM RF stock solution. Times of light exposure are numbered on the spectra as 1 to 10 and are, in minutes, 0 (1), 10 (2), 20 (3), 30 (4), 50 (5), 70 (6), 100 (7), 130 (8), 160 (9) and 190 (10).

reflecting a relatively dilute RF structure while photoproduct A is undergoing a reorientation (seen by the sharp anodic peak at ca. −0.5 V) indicative of a relatively condensed structure, suggests that RF and photoproduct A are present in discrete clusters on the electrode surface.

3.3. Identification of photoproducts

A probable photoproduct of the flavins is known to be lumichrome (LC) [3]. LC is not a member of the flavin family, due primarily to the change in distribution of the double bonds in the ring systems (Fig. 8) vs. that of the isoalloxazine system (Fig. 1). LC was reported in early work [3] to have an $E^{\circ\prime}$ more negative than that of the flavins. The LC redox reaction, which was studied earlier only by examining diffusion controlled electrochemistry, was claimed to be irreversible and without involvement of adsorption. To test for the formation of LC in the present experiments, a CV of a 3 μmol l^{-1} LC solution, buffered to pH 7, was obtained at a fresh Hg electrode (Fig. 9). An excellent match between the redox potential and peak shape of LC with that of product A in Figs. 4 and 5 is seen. The maximum charge density obtained at full coverage for LC was determined to be ca. 26 to 28 μC cm^{-2}, a little larger than in the case of LF (Table 1). The LC surface redox reaction is likely, therefore, to involve two electrons also. This relatively high surface coverage indicates that a high degree of order can be achieved in a LC monolayer, also consistent with the fact that the total charge density of a nearly fully photolyzed RF or FMN solution yields higher adsorption charge densities than observed for the original flavin solutions prior to photolysis. Further confirmation of photoproduct A as being lumichrome is seen from the LC spectrum (Fig. 10), which is very similar to that reported previously in the literature [36]. The main peaks are seen to be centred at 354 nm [35] and 260 nm, very similar to the developing bands in the spectra obtained for RF, FMN and FAD after photolysis (Fig. 7).

The identity of photoproduct B is more difficult to establish. The literature presents a number of possible photochemical reactions for the flavins [32–35,37]. One of these is photoreduction, which is known to occur both by

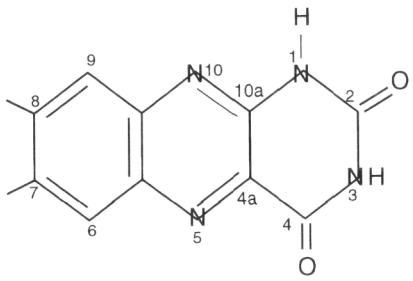

Fig. 8. The structure of lumichrome, the likely identity of photoproduct A.

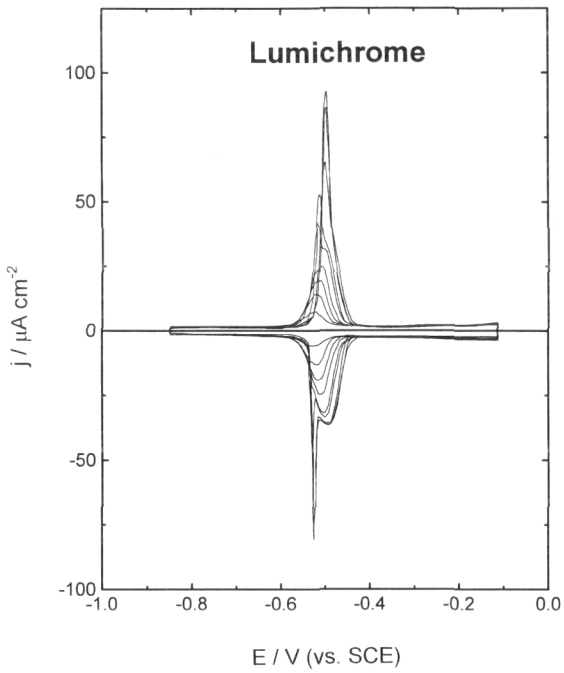

Fig. 9. The CV response of a fresh Hg electrode in a 3 μM lumichrome solution (pH 7) at 100 mV s^{-1}.

intermolecular and intramolecular modes. In all cases, flavin photoreduction can be observed spectrally only under anaerobic conditions [32], since in the presence of oxygen, the reduced flavin can be reoxidized. Since product B is seen electrochemically only when photolysis is carried out in the presence of oxygen, the photoreduction pathway in the generation of product B can be ruled out. It is also notable that one suggested photoreduction mechanism involves the generation of a flavosemiquinone radical [36], which would be expected to oxidize at a different potential from the original flavin; however, the lifetime of such a radical in aqueous solutions would probably be too short for it to be detected.

A second possible photoreaction involves a photodealkylation process [32,33], an intramolecular reaction yielding lumichrome, already identified as product A, as well as an alkene or substituted alkene. However, it is

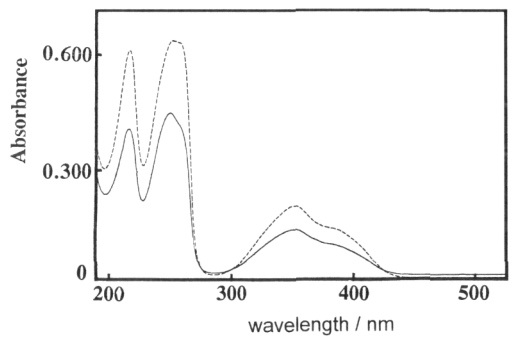

Fig. 10. Spectra of 7 (————) and 10 μM (---) stock lumichrome solutions.

unlikely that photoproduct B would be the alkene, as the product of its oxidation would be expected to be CO or CO_2 which, in turn, would not be expected to reduce at the flavin potential (Fig. 4).

A third possibility involves a photoaddition reaction [33,34], which is the most likely one to have yielded photoproduct B. Intramolecular photoaddition on C-9, in which the R group, i.e. the side chain, attached to N-10 attaches to C-9, yielding a fourth ring in the molecule, is expected to occur only if a hydroxyl group is present on the C-2 carbon of the R group. This is the case for FAD, RF and FMN (Fig. 1), for which products B (and A) are obtained. It is notable that the photoaddition product retains the isoalloxazine ring system, characteristic of the flavins, which would be consistent with the electrochemical reduction of B occurring at the expected flavin potential (Fig. 4); the positive shift of the oxidation of product B may be kinetic or steric in nature. Some further support for photoproduct B being the intramolecular photoaddition product comes from the fact that the formation of both products A and B is much slower for FAD than for RF and FMN. It would be reasonable to suggest that steric hindrance to internal cyclization would be more significant in the case of FAD, because of its lengthy sugar/phosphate/ribosyl/adenine chain attached to N-10. Spectroscopically, the photoaddition product has been reported to exhibit an absorption band in the 390 to 410 nm range [34]. This is in the range of the spectral feature which is shown in Fig. 7 to increase and then decrease in size, consistent with analogous changes seen in the CV response of product B.

While the above argument supports photoproduct B as being the product of the intramolecular addition of the side chain to the C-9 part of the isoalloxazine ring system, the literature also reports that the presence of divalent ions (e.g. HPO_4^{2-} or Mg^{2+}) is necessary for the photoaddition reaction to occur [34]. However, in our experiments, photolysis was successfully achieved, leading to both products A and B, in both phosphate solutions and in pure water, without a discernible difference in the amount of product B generated. Also, there is no mention in the prior literature that oxygen is a requisite for the intramolecular addition reaction, as observed in our work for the formation photoproduct B. While photoproduct B, in our view, is likely to be the product of intermolecular photoaddition, there is also mention in the earlier literature of another possible product of flavin photolysis, 7,8-dimethyl-10-formylmethylisoalloxazine [37]. Polarographic studies showed two irreversible reduction waves at neutral pH, which would fit the appearance of the oxidation peak of photoproduct B and the peak for its reduction, as shown in Fig. 4. However, we have no evidence to support this assignment of product B in the present work; rather, the intramolecular photoaddition product seems the most likely.

Acknowledgements

We gratefully acknowledge the Natural Sciences and Engineering Research Council of Canada for their support of this research. Also, we express our thanks to Dr. D.A. Armstrong for numerous helpful discussions and to C. Percy and M. Pinkerton for their technical assistance.

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