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Photoaddition of Alcohols and Ethers to Flavins in the Presence of EDTA

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Abstract: Alcohols and ethers that do not normally photoadd to flavins do so in the presence of EDTA to produce covalent attachment of an alkyl group at C(4a). Thus, irradiation of an aerated solution containing both EDTA and t-butanol resulted in rapid formation of an air-stable, reduced flavin, FlH-C(4a)$CH_2C(CH_3)_2OH$. Similar results were also obtained with ethanol, 1-propanol, 2-propanol, pinacol, poly(ethylene glycol), and dioxane, but not with methanol or acetone.

Flavin photochemistry has been extensively studied due in part to its proposed involvement in such biological phenomena as animal and plant photoreception and DNA repair enzymes.[1,2] Previous studies have demonstrated that flavins undergo a wide range of photoreactions, including photoreduction, electron transfer, N(10) dealkylation, photohydration and photoaddition.[3] Intermolecular photoreduction has been extensively studied in the presence of substrates such as amino acids, α-hydroxycarboxylic acids, thiols, aldehydes and unsaturated hydrocarbons.[3] Depending on the substrate and reaction conditions, photoreductions may lead to 1,5-dihydroflavin or N(5)-, C(4a)- or C(8α)-adducts.[4,5]

Unlike classical n-π^* triplet states such as ^{3}benzophenone, flavin triplets do not abstract hydrogen atoms from alcohols. Thus electron transfer and photoaddition remain as pathways for oxidation of alcohols, with concomitant reduction of the flavin. The extensively studied photoreduction of flavins by ethylenediaminetetraacetic acid (EDTA) proceeds via a primary electron transfer to the flavin in its triplet state,[6] followed by a secondary transfer from a decarboxylated EDTA radical to an oxidized flavin, with an optimal efficiency equal to the quantum yield of intersystem crossing.[7]

We now report that *t*-butanol, although unreactive towards flavins alone,[8] can alter the course of the normally reversible EDTA photoreaction to produce covalent attachment of the 2-hydroxy-2-methylpropyl moiety at C(4a). Certain other alcohols and ethers were found to react similarly. The reaction might therefore be a general method of introducing substituents that cannot be incorporated by the direct reaction with the flavin due to the low reactivity of the substrate.

Materials and Methods

Nuclear magnetic resonance spectra were recorded with a Varian Gemini 300 MHz spectrometer. Samples were dissolved in D_2O. Photoreactions were carried out at room temperature in aqueous solution at pH 4.4 (0.01 M phosphate buffer). The solutions for flavin photoreactions typically contained 10^{-4} M carboxymethyllumiflavin (cFl) or flavin mononucleotide (FMN), 0.01 M EDTA or oxalate, and 0.1 M alcohol. Reduction of the flavin was accomplished by irradiation of the air-purged solution with a 200-watt tungsten light bulb (filtered through glass) at a distance of approximately 8 cm. For a 3-mL volume, the

photoreaction was complete after 3 minutes of irradiation. Steady-state gamma irradiations were performed on a ^{137}Cs-γ-irradiator (Maintance Ltd). The dose rate was 1.5×10^{-2} Gy s^{-1} as measured by Fricke dosimetry.

Results and Discussion

Irradiation of cFl under anaerobic conditions in the presence of EDTA is known to result in rapid photoreduction to the 1,5-dihydroflavin and is 100% reversible upon aeration. When *t*-butanol (0.1 M) was included, under anaerobic conditions, irradiation also resulted in a rapid photoreduction of the flavin. Subsequent aeration, however, yielded only 70-80% of the original oxidized flavin absorption. Apparently an oxygen-stable species had formed.

We now report that irradiation of an aerated solution of cFl or FMN in the presence of both EDTA and *t*-butanol produced an alcohol-flavin adduct (see NMR below). Adduct formation was accompanied by a complete and permanent loss of the flavin absorption and the appearance of a new absorption at 360 nm (Figure 1). In contrast to adduct formation, irradiation without EDTA resulted in only an extremely slow loss of the flavin absorption. This meant that the direct reaction of excited flavin and *t*-butanol was inefficient. In addition, the adduct was produced if oxalate was included in place of EDTA.

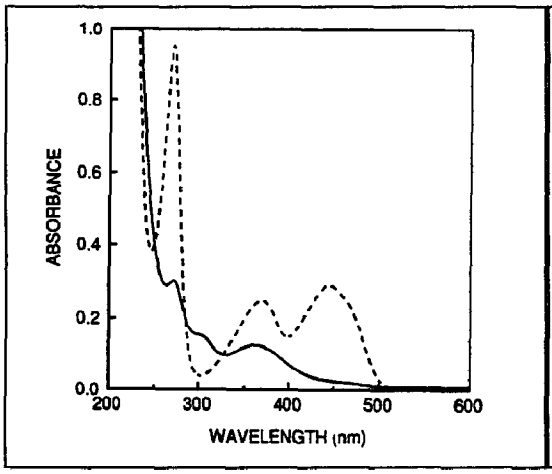

Figure 1. UV-visible absorption spectrum of cFl before (dashed line) and after (solid line) irradiation of the aerated solution containing 0.01 M EDTA and t-butanol.

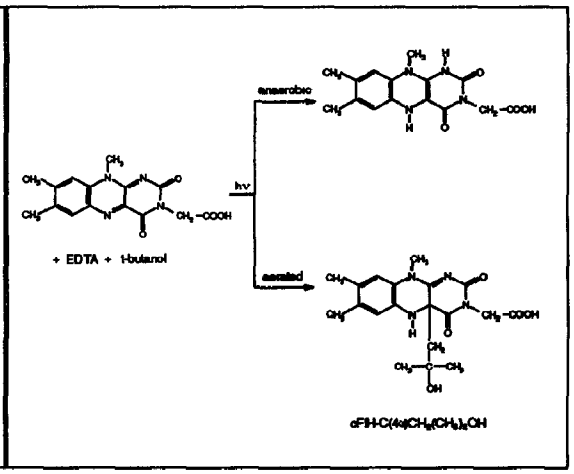

Figure 2. Structures of the 1,5-dihydroflavin and the oxygen-stable adduct formed by irradiation of cFl in the presence of EDTA (or oxalate) and t-butanol under anaerobic and aerated conditions.

NMR analysis of the product formed from cFl and *t*-butanol and oxalate showed that it was the C(4a) adduct shown in Figure 2. The product had the following NMR characteristics: δ (ppm) 1.02, s, 3H, C(4a)$CH_2C(CH_3)(CH_3')OH$; δ 1.13, s, 3H, C(4a)$CH_2C(CH_3)(CH_3')OH$; δ 1.4, d (J = 20 Hz), 1H, C(4a)C*H*H'; δ 2.0, d (J = 20 Hz), 1H, C(4a)CH*H*'; δ 2.19 and 2.12, 2 s, 6H, C(7)CH_3 and C(8)CH_3; δ 3.56, s, 3H, N(10)CH_3; δ 4.26, d (J = 16 Hz), 1H, N(3)C*H*H'; δ 4.34, d (J = 16 Hz), 1H, N(3)CH*H*'; δ 6.83, s, 1H, C(9)H; and δ 7.15, s, 1H, C(6)H. The signals at δ 1.02 and 1.28 are key to the determination of the structure as a C(4a)-substituted adduct. The geminal methyl groups are diastereotopic due to the new chiral center at C(4a), as are the protons of the CH_2 groups (signals at δ 1.4 and 2.0, and at 4.26 and 4.34). Thus, N(5)-alkylation can be ruled out

because it does not produce a chiral center. Also consistent with C(4a)-alkylation was the observation that the adduct's λ_{max} shifted to longer wavelength by approximately 30 nm in 6N HCl, which is known to be the case for C(4a) adducts but is not seen with N(5) adducts.[9]

Air-stable irradiation products that were almost identical spectrally to that formed with *t*-butanol were similarly formed with 1-propanol, 2-propanol, ethanol, pinacol, dioxane, and poly(ethylene glycol), but not with methanol or acetone. The reaction was found to proceed most rapidly with an aerated solution irradiated in a stoppered vessel. Possibly a low level of O_2 can then be present, which causes minimal quenching of triplet flavin yet still allows reoxidation of any 1,5-dihydroflavin formed. Under continuous aeration, the air-stable adduct is formed, but much more slowly.

It has been suggested that the primary electron abstraction of the flavin triplet from EDTA is followed by decarboxylation and then an internal hydrogen shift to produce the reducing radical[6] III, shown in Figure 3.

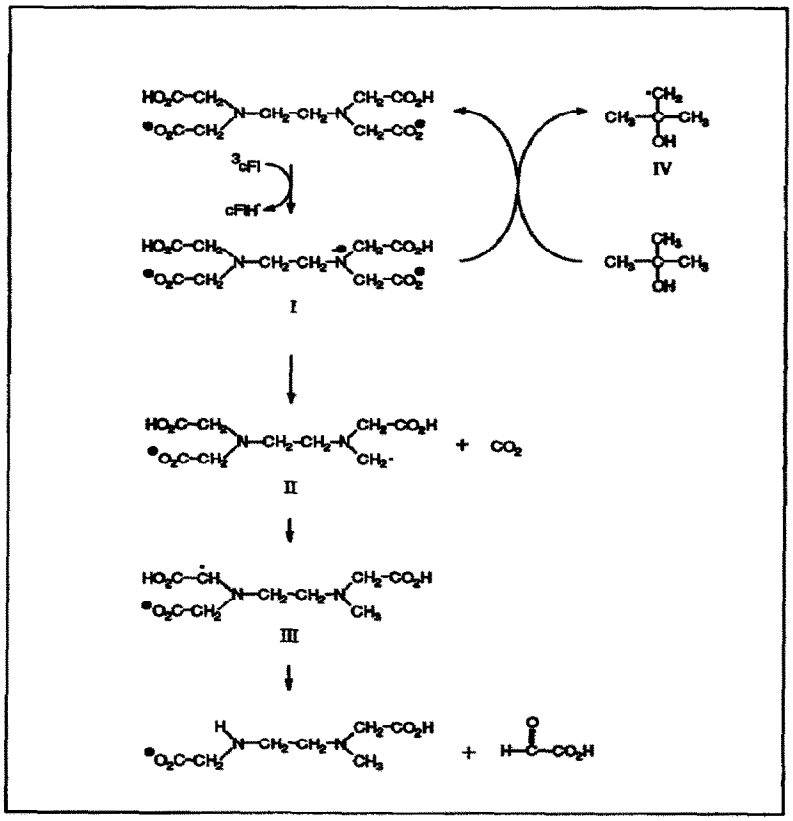

Figure 3. The species presumed to be intermediates in the photooxidation of EDTA by cFl. Radical I is responsible for the formation of $(CH_3)_2C(OH)CH_2^{\bullet}$ from t-butanol.

It is suggested that the alcohol reacts with the first-formed, oxidizing radical (I) or the decarboxylated species (II) via hydrogen atom abstraction from the β-carbon atom to produce the 2-hydroxy-2-methylpropyl radical (IV). The observation that the tetramethyl ester of EDTA, which cannot decarboxylate, also gives the photoreaction, is evidence for the involvement of

radical I. Addition of radical IV followed by hydrogen abstraction then produces the adduct. In fact, radical addition to oxidized and one-electron-reduced flavins has been documented previously[10,11] for β-hydroxy radicals (e.g. $\cdot CH_2CH(OH)CH_3$). In contrast, α-hydroxy radicals (e.g. $(CH_3)_2C\cdot$-OH) usually carry out a one-electron reduction instead of addition. The fact that methanol does not undergo the addition reaction is support for this mechanism. The ethers dioxane and polyethylene glycol can be envisioned to undergo hydrogen abstraction by an EDTA radical to produce an α-alkoxy radical that would add to the flavin.

Further support comes from the results of gamma radiolysis. In solutions saturated with nitrous oxide, the mixture of the primary products of radiolysis, namely H•, OH• and the hydrated electron, is converted to only OH•. Radiolysis of *t*-butanol in the presence of cFl under conditions where the former scavenges all of the OH• radicals results in the formation of a flavin compound that is not reoxidized by oxygen and has an absorption spectrum similar, but not identical to, the photoproduct with EDTA plus *t*-butanol. NMR spectroscopy, however, revealed the presence of a number of products. In this case it is known that OH• attack on *t*-butanol produces primarily (>95%) the β-hydroxy radical $(CH_3)_2C(OH)CH_2\cdot$.

Gamma radiolysis of cFl plus EDTA under conditions where the EDTA scavenges all of the •OH radicals does not result in the formation of any flavin products, as judged by the minimal change in the absorption spectrum. Therefore, although known to produce a variety of EDTA radicals, the oxidation of EDTA does not lead to any flavin addition products.

In contrast, gamma irradiation of cFl, EDTA and *t*-butanol under conditions where EDTA scavenges all of the OH• radicals results in the formation of an air-stable flavin compound almost identical spectrally to that observed upon radiolysis of cFl plus *t*-butanol. The NMR spectrum reveals the presence of a number of products. This demonstrates that OH• reaction with EDTA plus *t*-butanol can give products apparently similar to that for direct attack of $(CH_3)_2C(OH)CH_2\cdot$ on cFl. The profusion of products in the case of the radiolysis experiment is probably due to the fact that radicals derived from EDTA and *t*-butanol continue to be formed even after the flavin is completely reduced, possibly leading to secondary reactions. In contrast, in the photoreaction all the radicals are generated initially by oxidized flavin, and radical formation ceases upon flavin reduction.

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