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OXIDATIVE DEALKYLATION OF 4a-ALKYLATED 4a,5-DIHYDROFLAVINS

PROPERTIES OF 4a-ALKYLATED FLAVIN RADICALS

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Abstract—Covalent coenzyme substrate adducts ("σ-complexes") are probable intermediates in flavin-dependent biological dehydrogenations. As chemical model reaction for the σ-complex decay, oxidative dealkylation of stable 4a-alkyl-4a,5-dihydroflavins was studied as a function of alkyl mobility and nature of the oxidizing agent. The alkyl groups studied were n-propyl, allyl and benzyl, the oxidizing agents $^3O_2$, $^1O_2^*$, nitroxide radical, ferricyanide and light-excited flavin.

For all three alkyl residues, the primary reaction is formation of the 4a-alkyl-4a-hydroflavin radical by $1e^-$-abstraction. $^3O_2$ and ferricyanide are too weak to initiate this step. If, however, the radical 4a-RFl$^\cdot$ is once formed, at least five decay modes can be observed depending on the nature of R:

(1) For saturated R the exclusive decay is back transfer of the electron initially abstracted. In this case, dealkylation can only be obtained with $^1O_2^*$, albeit with the relatively slow rate of $< 10^6\ M^{-1}s^{-1}$.

(2) For unsaturated R further $1e^-$-oxidation leads to quantitative formation of oxidized flavin, while the fate of the alkyl group is still uncertain: In any case, ROH and the corresponding aldehydes as well as the dimers R_2 can be excluded as products.

(3) Further oxidation by $^3O_2$ again leads to a quantitative yield of oxidized flavin while the alkyl residues are converted to peroxy radicals. In an autocatalytic reaction they form the corresponding hydroperoxides with starting 4a-R-Fl$_{red}$H, leading to acrolein (R = allyl) or benzaldehyde (R = benzyl) as the major products.

(4) In the absence of further oxidant, slow intramolecular alkyl migration is observed leading to the stable 5-alkyl-1,5-dihydroflavin isomer.

(5) Competitively, alkyl migration occurs intermolecularly with the starting material as carbenium acceptor, resulting in formation of the stable 4a,5-dialkyl-4a,5-dihydroflavin and unsubstituted radical HFl$^\cdot$, which disproportionates.

In recent years it has become apparent that fully reduced flavin species other than 1,5-dihydroflavin (1,5-H_2Fl$_{red}$, "flavohydroquinone") may be relevant in flavin dependent biological substrate dehydrogenation. In the reduction of free[1,2] as well as protein bound[3] flavin (Fl$_{ox}$, "flavoquinone"), 4a-alkylated derivatives of the isomeric 4a,5-dihydroflavin (4a-R-Fl$_{red}$-5-H)[2,3] and 5-alkylated derivatives of normal 1,5-dihydroflavin (5-R-Fl$_{red}$-1-H)[4-6] have been observed or postulated[7,8] as intermediates.

Walker *et al.*[2] were first to describe the 4a,5 - dihydroflavin chromophor ($\lambda_{max} = 360$ nm, $\epsilon = 6200\ M^{-1}cm^{-1}$, as 4a-benzyl derivative). This compound was then found to undergo autoxidative photodealkylation, in contrast to the 5-RFl$_{red}$H isomers which are autoxidized smoothly in the dark, irrespective of the degree of saturation (mobility) of the alkyl substituent.[9]

From this and further studies it turned out that we have to distinguish four types of alkyl substituents with respect to the mode and ease of fixation and elimination to and from position 4a:

(1) Saturated alkyl residues or vinylic groups cannot be removed from C(4a) unless drastic oxidation is applied which would lead to partial deletion of the flavin skeleton.[10]

(2) More active alkyl groups such as allyl and benzyl will be stable under anaerobic conditions, but will be smoothly dealkylated upon oxidation (Eqn. 1). In the present paper we report on the mechanisms of this oxidative dealkylation which have been elusive so far.

$$\text{4a-R-Fl}_{red}\text{H} \xrightarrow{ox} \text{ROH} + \text{Fl}_{ox}. \qquad (1)$$

(3) Highly active alkyl groups such as α-methyl-benzyl or α,α-dimethyl-benzyl have been found to migrate easily between positions 4a and 5[11] and can be readily eliminated in both ways, i.e. protolytically as nucleophiles according to eqn (2) or upon oxidation as in Eqn (1).

$$\text{4a-R-Fl}_{red}\text{H} \xrightarrow{H^+} \text{RH} + \text{Fl}_{ox}. \qquad (2)$$

(4) If the 4a-substituent is bearing a function XH (such as X = O, NH) in position α, fragmentation according to equation 3[4] will efficiently compete with nucleophilic fixation and reelimination according to eqn (2). Most of the biologically relevant substrate residues belong to the latter group which accounts for the fact that the corresponding flavin-substrate "σ-complexes" can only be observed as short-lived intermediates by investigations of rapid kinetics.[4-6]

$$\text{4a-R'CH(XH)-Fl}_{red}\text{H} \longrightarrow \text{4a-R'-CH=X} + \text{H}_2\text{Fl}_{red}. \qquad (3)$$

In the present paper we want to show that, even with the less active alkyl residues, mobility can be achieved upon oxidation, i.e. in the radical state. The decay modes of the alkyl semiquinones thus obtained will be outlined.

RESULTS

Singlet oxygen-dependent dealkylation of 4a-alkylated-4a-5-dihydroflavins. Flavoquinone is known to be a photosensitizer producing singlet oxygen[12] by the energy transfer reaction $^3Fl^*_{ox} + {}^3O_2 \rightarrow Fl_{ox} + {}^1O_2^*$. Studying photodealkylation of 4a-R-Fl_{red}-derivatives in the presence of Fl_{ox} and $^3O_2$ requires, therefore, knowledge of their $^1O_2^*$-reactivity in order to distinguish the sensitized reaction from direct attack of $^3Fl^*_{ox}$ at the substrate. We find that $^1O_2^*$, generated in boiling benzene from diphenylanthracene peroxide, reacts three orders of magnitude more slowly with 4a-R-Fl_{red}H than with diphenylisobenzofurane, a standard $^1O_2^*$-acceptor. At room temperature the latter has been shown to react with $^1O_2^*$ with a rate constant of $10^9\ M^{-1}s^{-1}$.[13] Hence, we can estimate $^1O_2^*$-reactivity of 4a-R-Fl_{red}H to be characterized by a rate constant of about $10^6\ M^{-1}s^{-1}$ which is considerably smaller than the rates found for the reaction of $^3Fl^*_{ox}$ with electron donors. The latter have been shown to be in the range of $> 10^8\ M^{-1}s^{-1}$.[14,15]

Aerobic photodealkylation. In the dark, aerobic solutions of 4a-R-Fl_{red}H are stable. In the presence of light, however, 4a-allyl-Fl_{red}H and 4a-benzyl-Fl_{red}H are dealkylated while 4a-propyl-Fl_{red}H remains unchanged. The quantum yields for both photodealkylations are identical ($\Phi = 0.24$) and the kinetics of Fl_{ox} formation are sigmoidal (Fig. 1). The action spectrum shows a single band centered around 450 nm. When the mixtures had been preincubated with Fl_{ox} (20% of the total flavin), however, the action spectrum shows both absorption bands corresponding to the long wavelength transitions of Fl_{ox} and the lag phase in the kinetics of the reaction disappears (Fig. 1). Photodealkylation is inhibited by iodide (5×10^{-5} M), i.e. at concentrations that do not quench flavin fluorescence, suggesting that flavoquinone triplet ($^3Fl^*_{ox}$) is the reactive species.

To test this possibility further, we replaced Fl_{ox} by other dyes, such as methylene blue or eosine. These dyes were also productive in the photodealkylation reaction and the relative reaction rates paralleled those of the respective photodehydrogenations of EDTA (Table 1). Thus it appears that the rate of aerobic dealkylation is governed by the potential of the photosensitizer according to eqn (4):

$$Fl_{ox} \xrightarrow{h\nu} {}^1Fl^*_{ox} \longrightarrow {}^3Fl^*_{ox} \xrightarrow[\text{4a-R-Fl}_{red}\text{H}]{} H\dot{F}l + 4a\text{-R-}\dot{F}l. \quad (4)$$

With α,α-dideuterobenzyl - 4a,5 - dihydroflavin the kinetics of the reaction remained unchanged indicating that the rate limiting step does not involve an H atom abstraction at the α-methylene group.

Precise gaschromatographic determination of the product from 4a-benzyl-Fl_{red}H yielded a mixture of 85% benzaldehyde and 15% benzyl-alcohol together with quantitative formation of flavoquinone. Product distribution was found to be independent of both solvent polarity and the nature of the initiating oxidant. Addition of superoxide dismutase (1 mg, activity 3000 units) had no effect on either the rate of aerobic photodealkylation or on product distribution.

Dealkylation initiated by nitroxide as $1e^-$-donor. Since photodealkylation appeared to be initiated by $1e^-$-oxidation we tested $1e^-$-oxidants which might achieve oxidative dealkylation in the dark. Ferricyanide ($E_0' = +430$ mV)[16] proved to be ineffective, while with spirocycloexylporphyrexide ($E_0' = +690$ mV)[17] quantitative formation of Fl_{ox} from 4a-allyl-Fl_{red}H and 4a-benzyl-Fl_{red}H was observed within 30 seconds. Again no isotope effect could be observed when C^2H_2–C_6H_5 was substituted for CH_2–C_6H_5 in a 4a-benzyl-Fl_{red}H, while side chain products were exactly the same as in the light reaction. In the absence of oxygen, formation of Fl_{ox} was nevertheless rapid. We were, however, unable to identify the products derived from the alkyl side chain. Low molecular weight products, such as benzyl alcohol, benzaldehyde, benzoic acid, of dibenzyl could not be detected.

Anaerobic photodealkylation. With ferricyanide as final electron acceptor (instead of O_2), dealkylation was achieved upon anaerobic illumination. The quantum yield (0.40) increased and the kinetics of the reaction were no longer sigmoidal reflecting the absence of $^3Fl^*_{ox}$ quenching by triplet dioxygen. Again, Fl_{ox} was formed quantitatively but side chain products could not be identified.

Photolysis (illumination in the absence of added electron acceptors) leaves 4a - propyl - Fl_{red}H and 4a - allyl - Fl_{red}H unchanged while 4a-benzyl - Fl_{red}H undergoes a series of reactions. In the pH-range between pH 5 and pH 9 only 10–15% Fl_{ox} are formed as evidenced by the absorbance increases at 445 nm, along with spectral changes characterized by a new absorption maximum at 250 nm and a 10 nm blue shift in the long wavelength absorption of 4a-benzyl-Fl_{red}H at 364 nm (Fig. 2). Upon admission of air rapid formation of Fl_{ox} (10–15% of the

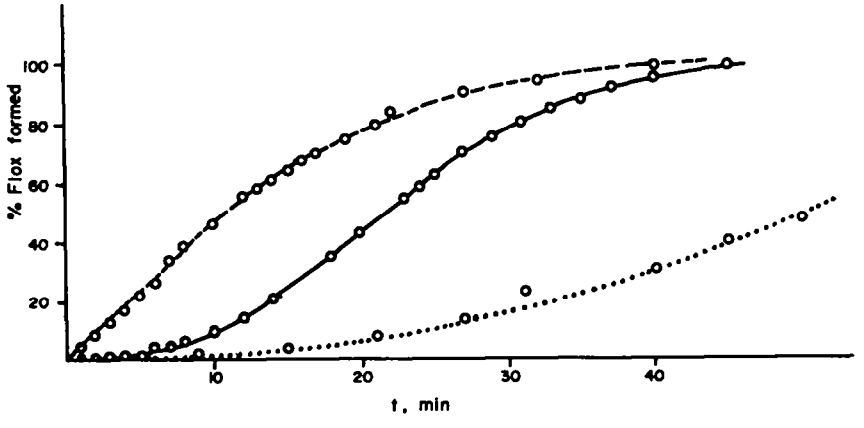


Fig. 1. Kinetics of photoautoxidation of 4a-benzyl-Fl_{red}H (10^{-4} M) in 0.1 M phosphate pH 7. (——): autocatalytic Fl_{ox}-formation without any addition; (– – –) disappearance of lag phase in the presence of 2.0×10^{-5} M Fl_{ox}; (···): inhibition in the presence of 5×10^{-5} M potassium iodide.

Table 1. Comparison of relative photooxidation rates between EDTA and 4-Bz-$Fl_{red}H$ with different dyes. Dye (10^{-5} M) in 0.1 M phosphate pH 7.0 containing 50% methanol (by volume) was photoreduced by EDTA (10^{-4} M) under anaerobic conditions. 4a-Bz-$Fl_{red}H$ (10^{-4} M) in 0.01 M phosphate buffer pH 7.0 containing 50% methanol (by volume) was photooxidized in the presence of dye (10^{-5} M) under aerobic conditions

Dye	relative rates for 4a-Bz-$Fl_{red}H$ oxidation	relative rates for EDTA oxidation
Fl_{ox}	1	1
eosine	1.5	1.5
methylene blue	7	10

total flavin) indicates that 1,5-H_2Fl_{red} had been one of the products. Concomitant slow formation of 5-benzyl-Fl$^{\cdot}$ (30–35% of the total flavin), identified by characteristic absorption spectrum,[2] shows that a second product must be 5-benzyl-$Fl_{red}H$ (Fig. 2). After its total conversion to Fl_{ox} with $NaNO_2$ in acetic acid a difference spectrum of the reaction mixture at this stage relative to Fl_{ox} is practically identical with that of authentic 4a,5-dibenzyl-Fl_{red} (Fig. 3), accounting for 30 to 35% of the total flavin. That 4a,5-dibenzyl-Fl_{red} had indeed been formed could be proven by thin layer chromatography (Fig. 4).

In an attempt to distinguish intramolecular from intermolecular alkyl-transfer a combination of water soluble 4a-benzyl-$Fl_{red}H$ (the 3-carboxy-methyl-derivative) and chloroform soluble Fl_{ox} was photolyzed and subsequently extracted with chloroform. If migration had occurred intra-molecularly, 5-benzyl-$Fl_{red}H$ should *only* be found in the aqueous phase. In case of intermolecular migration 5-benzyl-$Fl_{red}H$ should also be found in the chloroform phase. We found 5-benzyl-$Fl_{red}H$ in both the aqueous and chloroform phase in a ratio of 2:1. Thus it appears that benzyl migration proceeds via both, the intra- and intermolecular pathway.

The stability of 4a-*propyl*-$Fl_{red}H$. Since 4a-propyl-$Fl_{red}H$ is not oxidized by nitroxide in the dark, it could have a higher redox potential than the "activated" analogs. Such a property, however, cannot be responsible for the apparent photostability, as demonstrated by flash photolysis experiments. Following the absorbance of FlH$^{\cdot}$ at 590 nm we found identical yields for both 4a-propyl-$Fl_{red}H$ and 4a-benzyl-$Fl_{red}H$ indicating exactly the same extent of initial oxidation by $^3Fl^*_{ox}$. In either case the radicals formed after the flash had disappeared within two milliseconds. At that time, 4a-benzyl-$Fl_{red}H$ had been quantitatively converted to Fl_{ox}, while 4a-propyl-$Fl_{red}H$ was recovered unchanged. Thus, 4a-propyl-Fl$^{\cdot}$ radical must have been re-reduced by FlH$^{\cdot}$, whereas 4a-benzyl-Fl$^{\cdot}$ was rapidly dealkylated and/or oxidized. Hence, one has to conclude that nitroxide has a lower redox potential than the 4a-alkylated 4a,5-dihydroflavins investigated. The fast irreversible dealkylation of 4a-benzyl-Fl$^{\cdot}$ and 4a-allyl-Fl$^{\cdot}$ leads to quantitative formation of Fl_{ox} although only a small percentage of 4a-R-Fl$^{\cdot}$ radical is present at equilibrium.

DISCUSSION

Evidence obtained in a number of recent enzymological studies suggests that flavin-dependent dehydrogenation of biological substrates is a $2e^-$-process, involving labile covalent flavin-substrate intermediates.[3,4,6] Their chemical structure has to be interpreted as alkyl-dihy-

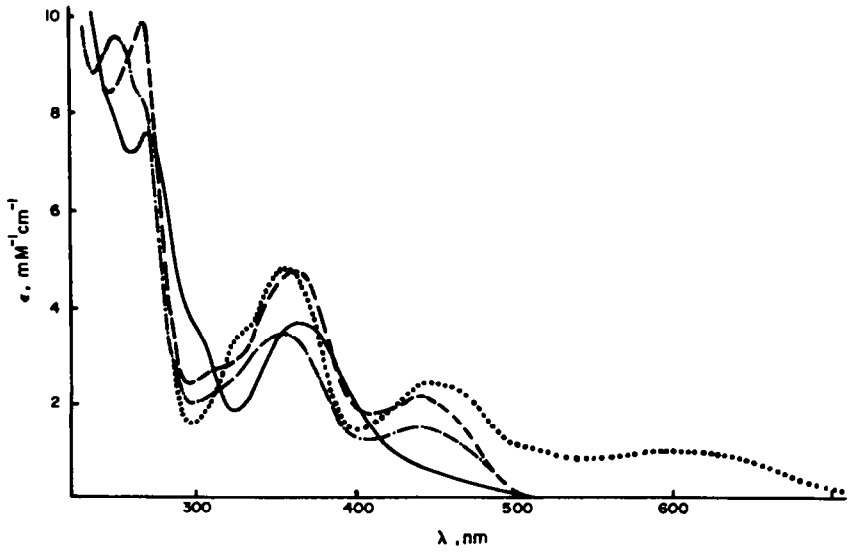


Fig. 2. (——): 10^{-4} M 4a-benzyl-$Fl_{red}H$ in 0.1 M phosphate pH 7; (– · –): reaction mixture after 60 min of illumination; (· · ·): transient formation of 5-benzyl-Fl$^{\cdot}$ after admission of air; (– – –): reaction mixture after complete air oxidation.

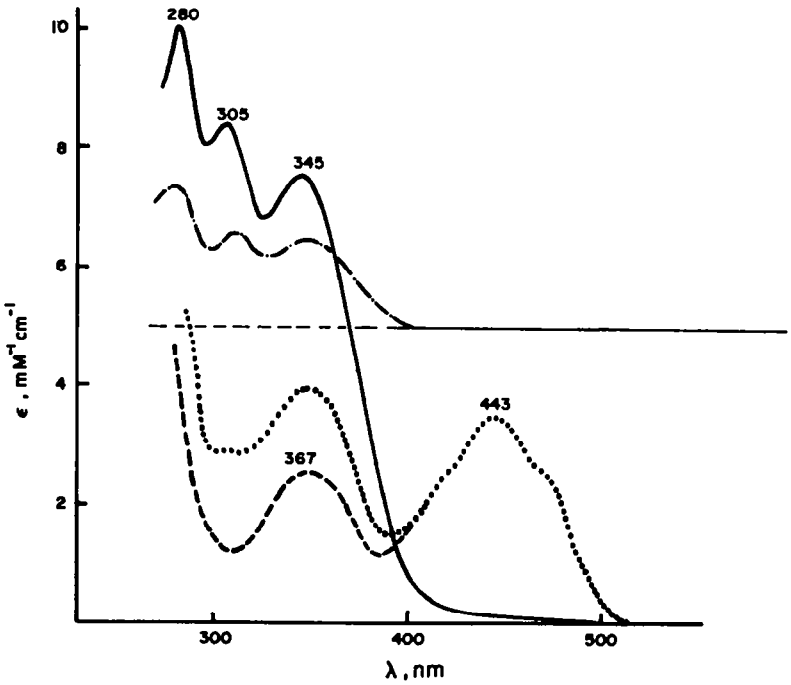


Fig. 3. (· · ·) reaction mixture after oxidation of 5-benzyl-$Fl_{red}H$ with HNO_2; (– – –) pure Fl_{ox}; (–·–) difference spectrum between pure Fl_{ox} and reaction mixture after HNO_2 oxidation; (——) 10^{-4} M 4a,5-dibenzyl-Fl_{red} in 0.1 M phosphate pH 7, authentic specimen [31].

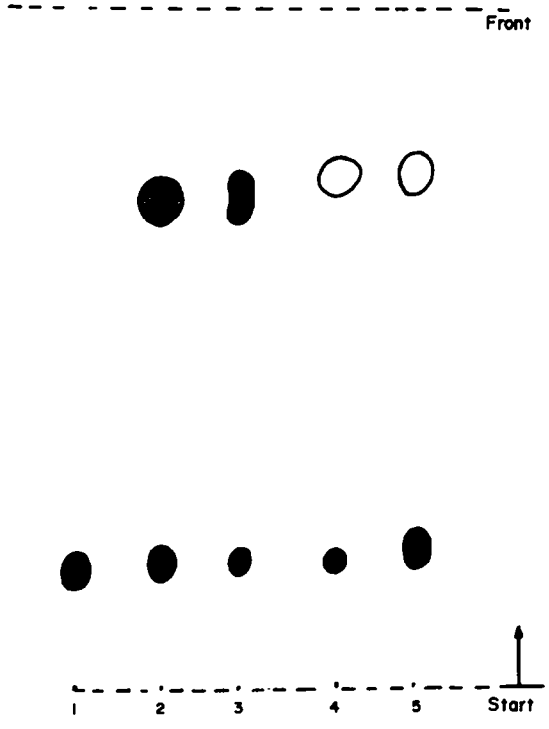


Fig. 4. Identification of 4a,5-dibenzyl-Fl_{red} as a product of anaerobic illumination of 4a-benzyl-$Fl_{red}H$ by thin layer chromatography (solvent system butanol/acetic-acid/water = 5/·3/2 by volume): 1 = Fl_{ox}; 2 = 4a-benzyl-$Fl_{red}H$; 3 = 5-benzyl-$Fl_{red}H$; 4 = 4a,5-dibenzyl-Fl_{red}; 5 = reaction mixture after oxidation by HNO_2. The shaded areas indicate fluorescence (UV-light λ_{max} = 254 nm) due to Fl_{ox} which is always present as an impurity. It is also formed from 4a-benzyl-$Fl_{red}H$ and 5-benzyl-$Fl_{red}H$ on the chromatographic plate when exposed to air.

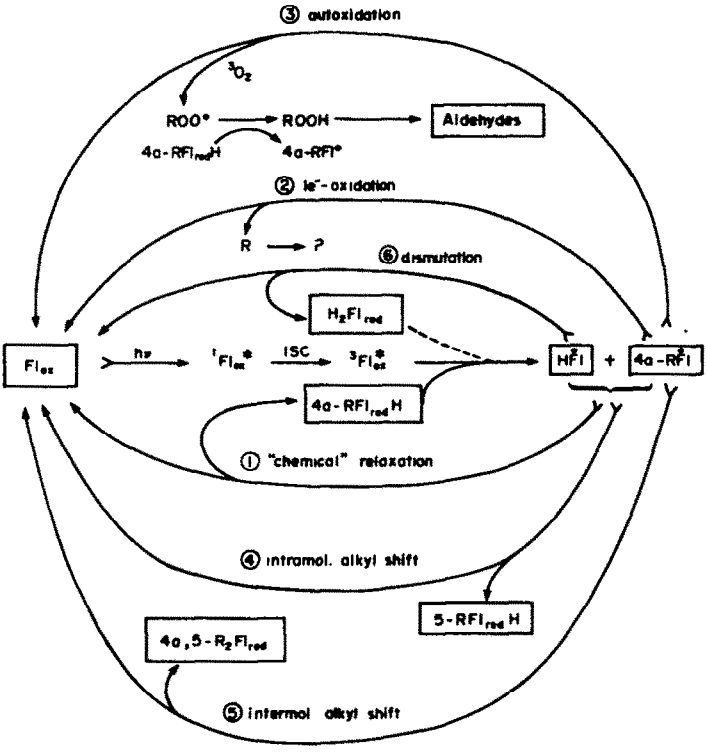
droflavins R-$Fl_{red}H$. The alkyl residues of biological relevance are mostly of the type > CX-, i.e. they bear a functional group (–NH_2 or –OH) in position α, and exhibit mobility already in the reduced state. This has also been shown with more labile residues earlier[11] when we demonstrated 4a-5-migrational aptitude in models R-$Fl_{red}H$, R being α-dimethyl-benzyl. The alkyl-dihydroflavins presented in this investigation are more stable, the alkyl-substituent can be mobilized, however, under oxidizing conditions:

The primary step consists of $1e^-$-oxidation, yielding an unstable radical 4a-R-Fl· which can decay in various ways. As $1e^-$-oxidants a stable radical of high energy, i.e. spirocyclohexylporphyrexide (E'_0 = 690 nm), or excited dye triplets, preferably $^3Fl^*_{ox}$ itself, proved to be effective while triplet dioxygen or ferricyanide were not. From this we can estimate a minimal redox potential for 4a-R-$Fl_{red}H$ of +700 mV. Once the radical 4a-R-Fl· has been generated, weaker oxidants—such as $^3O_2$, ferricyanide—can serve as secondary acceptors which at the same time induce cleavage of the R-Fl bond. Thus we distinguish the following decay pathways (Scheme 1) of 4a-R-Fl·.

(1) Reversal of the initial oxidation step through reduction of 4a-R-Fl· by FlH·. Similar flavin-dependent photooxidations followed by fast reversal in the dark have been described by Tollin *et al.*[14] for phenol and by Traber *et al.*[15] for the reaction of $^3Fl^*_{ox}$ with its own ground state. For 4a-propyl-Fl· this is the exclusive pathway of decay.

(2) $1e^-$-donation to ferricyanide or nitroxide radical, yielding Fl_{ox} and—presumably—carbene, followed by polymerization. This interpretation is based on the observation that no low molecular weight products could be observed with 4a-R-Fl· such as benzylalcohol, benzaldehyde, benzoic acid or dibenzyl.

(3) $2e^-$-donation to O_2, yielding Fl_{ox}, aldehyde and



Scheme 1.

alcohol. This pathway involves the intermediate formation of a labile primary alkylhydroperoxide. Aldehyde and alcohol are well established breakdown products of such hydroperoxides.[18,19]

(4) Alkyl migration, yielding 5-R-Fl$^{\cdot}$ and, after reduction by FlH$^{\cdot}$, 5-R-Fl$_{red}$H.

(5) Alkyl transfer, yielding $Fl_{ox} + R_2\text{-}Fl_{red}$. The last two reactions are only possible with the very labile benzyl substituent. They are at least 100 times slower than reactions (1)–(3).

As shown in Scheme 1, there are two autocatalytic steps in the dealkylation reactions: in reactions (2)–(4) the final product Fl_{ox} is at the same time photosensitizer of the initial step. In reaction (3) the alkylperoxy radical is an intermediate of strong oxidizing power towards the starting 4a-R-Fl$_{red}$H. This autocatalytic step is well established for the autooxidation of hydrocarbons.[20]

Summarizing, substrate residues R *in alkylated dihydroflavins* should be divided into three classes of decreasing mobility depending on the α-substituents in R:

(a) Functional, protic α-substituents such as –OH and $-NH_2$ permit fragmentation of R as shown in eqn (5):

$$>C(XH)\text{-}Fl_{red}H \longrightarrow >C{=}X + H_2Fl_{red}. \qquad (5)$$

(b) Residues R with π-electron containing α-substituents, such as phenyl and vinyl, can either migrate or be cleaved after 1e$^-$ oxidation as shown in eqn (6).

$$>CAr\text{-}Fl_{red}H \xrightarrow{1e^-} >CAr\text{-}Fl^{\cdot} \longrightarrow \overset{+}{C}\text{-}Ar + \overset{\cdot}{F}l^- . \qquad (6)$$

(c) Fully saturated residues R cannot be removed at ambient temperature. This reminds somewhat of the transition of bond nature in simple cyclopentadiene derivatives R-C_5H_5 from fixed (R=CH_3) to floating σ-bonds (R = $Si(CH_3)_3$) and finally to π-bonds R = FeC_5H_5 and ion pairs (R = Na).[21]

The following conclusions of biological relevance can thus be drawn

(1) Even if radicals are involved in the decay of a flavin-substrate complex, dioxygen will not necessarily act as 1e$^-$-acceptor. We could not detect formation of superoxide which is in agreement with recent data of Kemal *et al.*[22] Instead, a substrate peroxide is assumed which would yield preferentially aldehyde by rearrangement. Hence, the total (photo)reaction of 4a-R-Fl$_{red}$H with O_2 appears to be a "mixed function oxygenation", yielding oxygenated substrate and water, but no hydrogen peroxide. This should be seen together with the now well established reaction of flavin-dependent bacterial luciferase, where aldehyde is the substrate and acid the product, involving the decay of a ternary complex:

$$R\text{–}CH\begin{pmatrix} O\text{–}O \\ Fl_{red} \end{pmatrix} \longrightarrow Fl_{ox} + RCOOH^{\cdot} \qquad (7)$$

(2) A once formed flavin radical is not necessarily a candidate for further oxidation or reduction. Instead, rearrangements and transfer reactions may occur at the radical level, followed by "interflavin" electron exchange (dismutation). In several enzyme systems, interflavin reactions appear to be favored by protein structure and/or conformation.[23,24]

(3) Flavin is an active surface, upon which even alkyl residues stable in themselves may undergo migration or oxidoreduction e.g.

$$Fl_{ox} + RH \rightleftarrows 4a\text{-}RFl_{red}H \rightleftarrows 5\text{-}RFl_{red}H \rightleftarrows [R^+] + Fl_{red}H^- \quad \text{e.g. } R = NAD \qquad (8)$$

(4) Flavin is a redox-system which is capable of dealing equally well, in principle, with $1e^-$- and with $2e^-$-equivalents in chemical or photochemical as well as in biological oxido-reduction. The actual mode of reaction is, in a chemical system, dictated by the nature of the substrates, donors ("CH-substrates") as well as acceptors (O_2 or Fe^{III}). In biological systems, the apoprotein decides which type of compound will, specifically, be substrate of a given flavo-protein by directing hydrogen bonds towards the chromophor, either in the region N(1)/O(2α) for $2e^-$-transfer, or in the region N(5) for $1e^-$-transfer.[25]

EXPERIMENTAL

3 - Methyl - 4a - benzyl - 4a,5 - dihydroflavin, 3 - methyl - 4a - allyl - 4a,5 - dihydroflavin, 3 - methyl - 4a - propyl - 4a,5 - dihydroflavin, 3 - methyllumiflavin, and 3 - carboxymethyl - lumiflavin were synthesized according to published procedures.[26,27] 3 - Methyl - 4a - α - dideuterobenzyl - 4a,5 - dihydroflavin was synthesized from α-dideutero - phenylacetic acid[28] and 3 - methyllumiflavin at 50°C, 3 - carboxymethyl - 4a - benzyl - 4a,5 - dihydroflavin from 3 - carboxymethyllumiflavin and phenylacetic acid at 50° according to Walker *et al.*[2] 9,10 - Diphenylanthraceneperoxide ("DAP") was a gift from Dr. W.-R. Knappe in this laboratory, spirocyclohexylporphyrexide was synthesized according to Porter and Hellermann.[17] All other chemicals and reagents were commercial products of the best available purity, they were used without further purification. Superoxide dismutase was purchased from Sigma, München.

Spectrophotometric measurements were made with a CARY 14 instrument, Thunberg type 1 cm cells were used for anaerobic measurements. Anaerobiosis of solns was achieved with deoxygenated argon. For photo-reactions a projector 250 W 24 V tungsten halogen light source was used, equipped with narrow band interference filters of the desired wavelength (band halfwidth 10 nm) and a heat filter. Action spectra were determined with a Zeiss M 4 Q3 monochromator equipped with a Xenon light source. Quantum yields were determined by a method similar to that of Kling *et al.*[29] Gaschromatographic analysis was performed with a Perkin Elmer F 21 preparative gas chromatograph using SE 30 analytical columns for detection of benzyl alcohol and tenzaldehyde. Singlet oxygen was generated chemically by refluxing a solution of "DAP" in benzene according to Wassermann *et al.*[30] 1,3 - Diphenylisobenzofurane was used as a standard for determining singlet oxygen reactivity[11] by following the decrease in absorbance at 420 nm ($\epsilon = 1{,}7 \times 10^4\ M^{-1}\ cm^{-1}$). The rate of DAP decay was obtained by following the increase in absorbance at 394 nm (production of 9,10-diphenylanthracene: $\epsilon = 10^4\ M^{-1}\ cm^{-1}$). In a typical experiment a 10^{-4} solution of 4a-alkylated-4a,5-dihydroflavin in benzene, containing a 500 fold excess of DAP was refluxed for approximately 24 hr. Taking samples at regular time intervals the rate of Fl_{ox}-formation was followed spectrophotometrically by the increase of absorption at 470 nm.

For flash photolysis experiments a type KR 1 instrument from Applied Photophysics, London was used, equipped with a Zeiss M 4 Q III-mono-chromator, a 250 W Xenon lamp and a Tectronix 5103 N oscilloscope. Cylindrical pyrex cells, light path 10 cm, were used.

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