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# ONE AND TWO ELECTRON TRANSFER PATHWAYS IN THE PHOTOREDUCTION OF FLAVIN †

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Abstract - The stoichiometry of flavin photoreduction was studied by flash photolysis, monitoring the conversion of photoexcited flavin to one-electron and two-electron reduction products and following their fate. The reactivity of the photoexcited states of flavin in one-electron transfer reactions was determined, the reasons for differences between them are discussed and the mode of radical decay was investigated in dependence of the photosubstrate structure. The nature of the two-electron transferring photosubstrate was revealed in order to define the range of the two reduction pathways.

## INTRODUCTION

The yellow, tricyclic flavin molecule (10-alkyl-7,8-dimethylisoalloxazin) is the chemically active cofactor of the flavoproteins, which are indispensable components in cellular redox metabolism and in several photobiological processes. A very important, and among biomolecules practically unique, feature is their ability to split electron pairs, removed from nicotinamide or carbanionic substrate residues, to single electrons and to transfer them to iron-sulfur or heme electron stores (Ref. 1).
The flavin chromophore has a chinoid electronic structure and therefore can accept an electron pair in two ways: either by a twofold uptake of single electrons via the flavosemiquinone form (Eq. 1) or by a two-electron reduction, in which a substrate makes its electron pair at once available to the flavin. The latter mechanism represents a nucleophilic addition of the substrate to the π-electron system of the flavin molecule. This flavin-substrate-adduct can now be split homolytically to yield the free flavohydroquinone form and the oxidized substrate (Eq. 2).

$$Fl_{ox} \xrightarrow{e^-,H^+} HFl^{\bullet} \xrightarrow{e^-,H^+} H_2Fl_{red} \qquad (1)$$

$$Fl_{ox} + RH \longrightarrow R\text{-}HFl_{red} \xrightarrow{H_2O} ROH + H_2Fl_{red} \qquad (2)$$

In comparison to other biological redox catalysts, flavoproteins are distinguished by the fact that they can facily undergo both one and two electron transfer pathways. The pathway is regulated by the protein-part of the enzyme, forming hydrogen bridges with or bringing positive charges to either the N(1)- ($2e^-$-transfer) or the N(5)-site ($1e^-$-transfer) of the flavin molecule (Ref. 2).
We will show that this versatility, not easily obtained in the protein-free flavin ground state, is also inherent to the photoexcited states of flavin.

† Dedicated to Professor Albert Weller for his 60th birthday.

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The transfer pathway is now regulated by the photosubstrates only, the properties of which will be described in detail.

## THE ONE-ELECTRON TRANSFER

### A. The primary process

Leonhardt and Weller (3) were the first to study electron transfer reactions in the photoexcited state by using the flash photolysis method. The system investigated was perylene in the excited singlet state and anilines, in which they observed perylene anion radicals. Since then the mechanism of the electron transfer reaction has been studied in many laboratories, the electron-donor-acceptor components being organic (Ref. 4,5,6,7), inorganic (Ref. 8,9,10) and compounds of special biological interest (Ref. 11,6). The electron transfer in the photoreduction of organic dyes has been studied extensively and is reviewed by Meier (12), Koizumi and Usui (13), Chibisov (14) and Koizumi et al. (15).

Beginning with the observation of Oster and Wotherspoon (16) in 1957 that photoexcited methylene blue is only reduced by secondary and tertiary, but not by primary amines, several attempts have been made to relate the photoreducing power of a substrate to its chemical structure (see for example Ref. 17). Again Rehm and Weller (18,19) were the first to correlate quantitatively the kinetic rate constants of the fluorescence quenching of polycyclic aromatic compounds by amino and methoxy substituted benzenes with the thermodynamic free enthalpy of reaction of the electron transfer process. They obtained a relation, the characteristic graphical shape of which is shown in Fig. 1, for the flavin photoexcited singlet quenching process and which is described mathematically by the following equations based on their reaction scheme in Eq. 3.

$$D^* + R \underset{}{\overset{k_{12}}{\rightleftharpoons}} \underbrace{D^* \ldots R}_{\text{encounter complex}} \overset{k_{23}}{\rightleftharpoons} \underbrace{{}^2D^{\bullet+} \ldots {}^2R^{\bullet-}}_{\text{radical pair}} \xrightarrow{k_{30}} \text{products} \qquad (3)$$

$$k_q = k_{12}/(1+(k_{21}/k_{30})\,[\exp(\Delta G_{23}/RT) + \exp(\Delta G_{23}^{\neq}/RT)]) \qquad (4)$$

$$\Delta G_{23} = FE_R^{ox} - FE_D^{red} - E_{o,o}(D^*) + (z-1)Fe_o/(4\pi\epsilon\epsilon_o a) \qquad (5)$$

$$\Delta G_{23}^{\neq} = (\Delta G_{23}/2) + [(\Delta G_{23}/2)^2 + (\Delta G_{23}^{\neq}(0))^2]^{1/2}$$

D is the electron acceptor dye, R the electron donating photosubstrate and $k_q$ the overall quenching rate constant. The reaction free enthalpy of the electron transfer process in the encounter complex is calculated by the difference of the oxidation and reduction potentials of the components ($E_R^{ox}$, $E_D^{red}$, F = Faraday constant), the excitation energy of the dye ($E_{o,o}(D^*)$) and the Coulomb energy required to bring the ion pair to the distance of the encounter complex (z = charge number of the dye, $e_o$ = elementary charge, $\epsilon$ = dielectric constant, $\epsilon_o$ = $\epsilon$ of vacuum, a = transfer distance, 7 Å). $\Delta G_{23}^{\neq}(0)$ is the activation free enthalpy for a reaction with $\Delta G_{23} = 0$.

Several alternative free enthalpy relations have been proposed, known as the Polanyi, Marcus and Scandola-Balzani equations, for the determination of the activation free enthalpy. The range of validity of these relations has been critically reviewed by Scandola et al. (20,21) and Shizuka et al. (22).

According to Rehm and Weller the electron transfer process is nearly diffusion controlled for very negative $\Delta G_{23}$-values ($k_q \approx k_{diff}$ for $\Delta G_{23} \lesssim$ -0.43 eV), the quenching rate constant then decreases with increasing $\Delta G_{23}$, when $\Delta G_{23}$ is around zero, and in the boundary region of highly endergonic reactions ($\Delta G_{23} \gtrsim 0.22$ eV) $k_q$ decreases exponentially with increasing $\Delta G_{23}$. The latter follows directly from Eq. 4, when $\Delta G_{23}^{\neq}$ is equated with $\Delta G_{23}$.

Equation 4 then reduces to Eq. 7:

$$k_q = (k_{12}k_{30}/2k_{21})\exp(-\Delta G_{23}/RT) \quad (7)$$

Taking the logarithm and substituting the oxidation potentials of the electron donors according to Eq. 5 for $\Delta G_{23}$ in Eq. 7 one obtains Eq. 8:

$$\log k_q = C - FE_R^{ox}/(2.3\ RT) \quad (8)$$

This equation predicts a straight line with a slope of $-17.0\ V^{-1}$, when $\log k_q$ is plotted versus $E_R^{ox}$. In electron transfer processes the position of this line on the scale of oxidation potentials defines the reactivity of the electron accepting, photoexcited dye.
Quenching rate constants of excited singlet and triplet states of flavin have been determined according to the Stern-Volmer method with methyl and methoxy substituted benzenes and naphthalenes as electron donors in methanolic solution (Ref. 24). The donors were selected so that adduct formation with the photoexcited flavin or energy transfer did not occur. The results are shown in Fig. 1, in which lines with the theoretically required slope are drawn through the experimental points.

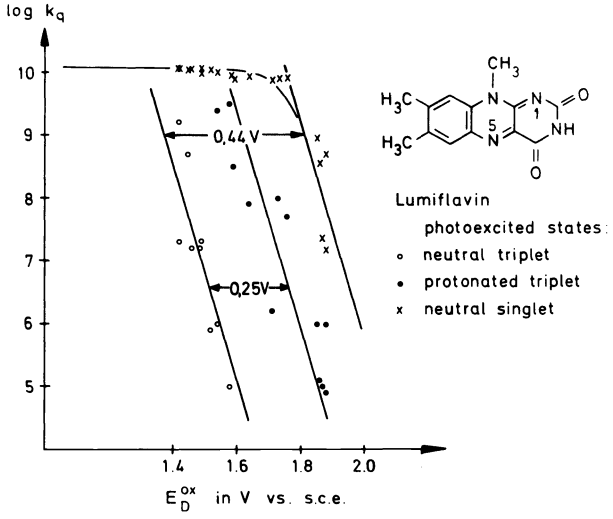



Fig. 1. The dependence of rate constants of flavin photoexcited state quenching on the one-electron oxidation potential of the donors in methanolic solution. Reproduced from Ref. 24 by courtesy of Pergamon Press Ltd..

The difference in reactivity of protonated and neutral flavin triplet states. As can be seen in Fig. 1, the reactivity line for the protonated flavin triplet is found to be at higher oxidation potentials than that of the neutral triplet. The reason for this can be shown to be due to the higher reduction potential of the protonated form as follows from the Michaelis cycle (Ref. 25,26). Applying the cycle to the flavin system the difference of the reduction potentials of the protolytic flavin triplet forms is related to the pK difference between the flavin triplet ($pK_T = 6.5$ in methanol (Ref. 24)) and the flavosemiquinone ($pK_S = 11.3$ in methanol (Ref. 24)):

$$FE^{red}({}^3HFl_{ox}^{+*}) - FE^{red}({}^3Fl_{ox}^{*}) = 2.3\ RT\ (pK_S - pK_T) \quad (9)$$

Using Eq. 7, the difference of the reaction rate constants is now determined thermodynamically (Eq. 10) and can be calculated by use of Eq. 5

$$\log k_q(^3HFl_{ox}^{+*}) - \log k_q(^3Fl_{ox}^{*}) =$$

$$- (2.3\ RT)^{-1}(\Delta G_{23}(^3HFl_{ox}^{+*}) - \Delta G_{23}(^3Fl_{ox}^{*})) \qquad (10)$$

The difference of the reaction rate constants corresponds to the vertical distance between the reactivity lines and indicates the magnitude by which the protonated triplet is quenched more by a given electron donor than is the neutral triplet. For our purpose it is more useful to know how much lower the potential of the electron donor must be to quench the neutral triplet to the same extent as the protonated triplet form. From Eq. 10 a decrease of 0.22 eV of the reaction free enthalpy for the neutral triplet reaction is predicted in order to obtain the same quenching rate constant. This corresponds to a theoretical horizontal displacement of the lines of 0.22 eV/F = 0.22 V, which agrees well with the experimental value of 0.25 V in Fig. 1.
In this connection it must be pointed out, that the pH-dependency of flavin triplet quenching will disappear for electron donors with oxidation potentials lower than 1.4 V vs. s.c.e., since now both protolytic triplet forms react with the diffusion controlled rate constant.
The difference in reactivity of protolytic triplet forms in one-electron transfer reactions explains the pH-dependency of many flavin photoreactions ("photo-pK", see Ref. 27) and has found an application in the pK-determination of the 5-deazaflavin triplet (Ref. 24).

The difference in reactivity of photoexcited flavin singlet and triplet states. Differences between singlet and triplet photoexcited states of dyes in the efficiency of electron abstraction and ground state deactivation have been studied by Joussot-Dubien and collaborators (28,29). In our case the difference in the overall quenching rate constants of photoexcited singlet and triplet states is considered. Applying the concept of Rehm and Weller, based on the photoexcited singlet state, to the triplet state too, the difference in reactivity should depend only on the difference in the excitation energies. Taking the singlet-triplet splitting energy of flavin (Ref. 30), a difference in the reaction free enthalpies of photoexcited flavin singlet and triplet states of 0.31 eV is calculated. From this value a theoretical horizontal shift of the neutral triplet quenching line for 0.31 V to lower oxidation potentials is expected with respect to the photoexcited singlet quenching line in Fig. 1. However, a distance of 0.44 V is found experimentally. From the point of view of the photoexcited singlet state, the reactivity of the flavin triplet is for 0.13 V lower than expected from the singlet-triplet energy splitting. For 5-deazaflavin a value of 0.13 V (from Ref. 24 with the excitation energies of Song et al. (31)) and for acridine orange a value of 0.12 V (Ref. 32) was obtained. The lower reactivity of the triplet state as compared to the photoexcited singlet state, exceeding the ΔG-corrected value, has been reported also for thionine (Ref. 25), methylene blue and o-methylfluorescein methylester (Ref. 33). Possible falsifications have been excluded, using the same set of electron donors for singlet and triplet quenching experiments and taking the data of the excitation energies all of which were determined under low temperature conditions.
A possible explanation of the effect can be offered, extending the interpretation of the reaction scheme proposed by Rehm and Weller, if we assume, that instead of a clearly solvent shared radical pair, an exciplex is formed in endergonic electron transfer processes (Eq. 11).

$$D^* + R \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \underset{\text{encounter complex}}{D^* \ldots R} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \underset{\text{exciplex}}{(\dot{D}^- \ldots \dot{R}^+)^*} \xrightarrow{k_{30}} D + R \qquad (11)$$

$$\downarrow k_{34}$$

$$\underset{\text{free radicals}}{\dot{D}^- + \dot{R}^+}$$

From this the following facts emerge:

1. The interaction in the exciplex will slow down the dissociation into free radicals and therefore alternative exciplex decay processes will dominate.
2. In endergonic overall electron transfer reactions the exciplex decay to the encounter complex is exothermic and therefore can compete effectively with the deactivation to the ground state.
3. Since it is spin-forbidden, the deactivation to the ground state is slower in the triplet exciplex than it is in the singlet exciplex. Therefore, the exciplex decay to the encounter complex is more important in the triplet than in the singlet exciplex.
4. The exciplex decay to the encounter complex leads back to the photoexcited state of the dye, and therefore this process diminishes the rate of the overall quenching reaction.

The consequence of these facts is shown experimentally by the difference in the quenching rate constants for electron transfer reactions which are equal in the reaction free enthalpy, as calculated from Eq. 5, but differ in the multiplicity of the photoexcited state. Vice versa, to obtain the same quenching rate constant in photoexcited singlet and triplet reactions the oxidation potential of the electron donor in the triplet reaction has to be even lower than calculated from the difference in excitation energies. This is observed in Fig. 1, explaining the additional shift of 0.13 V, mentioned above.

Thus the longer lifetime of a triplet exciplex, in which the back reaction can effectively compete with other decay processes in contrast to singlet exciplexes and radical pairs, will offer an explanation of the above observations in terms of generally accepted theories.

It should be pointed out that, from the experiments, an exciplex as intermediate is only required for endergonic electron transfer reactions. Very recently it was shown that exciplexes and solvent shared radical pairs are formed competively with the branching ratio between them being dependent on the value of the reaction free enthalpy of the electron transfer process (Ref. 34).

B. The secondary process

At this stage of our description of the efficiency of flavin photoreduction by a substrate in one-electron transfer reactions we now know the energetic requirements for the particular photoexcited states of flavin, which will yield a light-induced charge separation. But there are still two parameters to be discussed. The yield of free radicals and the yield of reduced flavin with respect to the free radical production.

The yield of free radicals is determined by the competition, occurring in both the exciplex and radical pair, respectively, between the rate of charge re-transfer leading to the ground state and the dissociation to free radicals (Ref. 35,36,37). Therefore, in photoexcited singlet state reactions, in which the spin-allowed deactivation to the singlet ground state is fast, very low radical yields are observed, while in photoexcited triplet state reactions, in which the spin-forbidden deactivation process is slow, high radical yields usually are obtained (Ref. 28,29,22). Steiner and collaborators (35,36,37,38) showed that the intersystem crossing process can be catalyzed by spin-orbit coupling properties of the photosubstrate radical, produced in the preceding charge transfer process.

Starting from the radical state, the yield of the reduced dye is determined by the competition between the electron back transfer, a reaction which is energetically strongly favored in light-induced charge separation reactions, and the processes which are capable of preventing the electron back transfer by rapidly changing the redox properties of the transient radicals. With respect to the substrate, the latter being deprotonation or decarboxylation processes, which eliminate the positive charge in the cationic substrate radical.

Examples for flavin photosubstrates yielding a "chemical deactivation" of the flavin excitation energy by complete electron back transfer are the nitrite anion and the 1,4-diazabicyclo[2,2,2]octane (DABCO) molecule (Ref. 39,40). The one-electron oxidation products are nitrogen dioxide and the DABCO radical cation. Nitrogen dioxide is known to be a rather stable radical and to be an oxidant and not a reductant. The DABCO radical cation is stabilized by electron delocalisation over the two bridge-head nitrogen centers and for this rigid, tricyclic N-amine radical it is not possible to deprotonate or dismutate, since it cannot assume a $sp^2$ configuration. Therefore the fate of both substrate radicals is confined to 100 % back electron transfer in presence of the reductant flavosemiquinone.

In Fig. 2 the flash photolysis of the flavin/nitrite system in aqueous phosphate buffer of pH 7 is shown. Just after the flash the neutral flavosemiquinone appears with a maximum of absorbance at long wavelength at 560 nm. The flavosemiquinone decays concomitantly to the re-formation of the flavoquinone, absorbing at 440 nm. According to Eq. 12 an isosbestic point is found at 483 nm in the reaction. Since the absorbances of the nitrite ion and nitrogen dioxide are very small at this wavelength, it is the isosbestic point between the flavoquinone and the flavosemiquinone. The usefulness of this fact will become clear below.

$$Fl_{ox} + NO_2^- \underset{}{\overset{h\nu, +H^+}{\rightleftharpoons}} \dot{H}Fl + NO_2 \qquad (12)$$

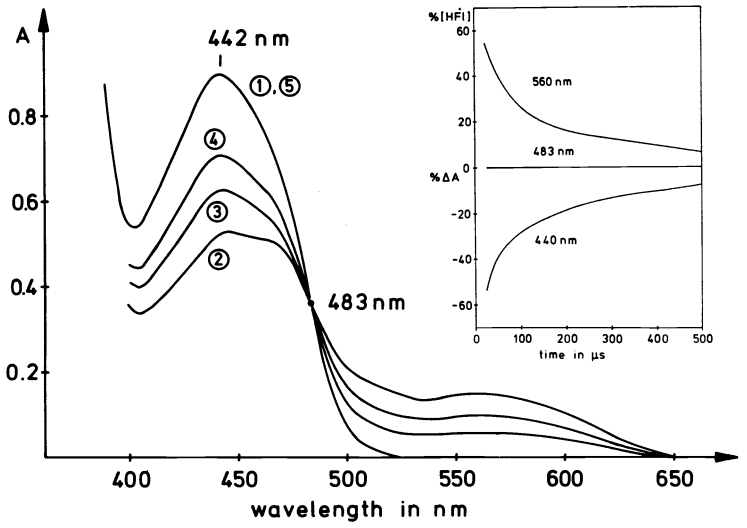



Fig. 2. The reversible one-electron photoreduction of 3-methyllumiflavin (7.5 µM) by sodium nitrite (0.01 M) in anaerobic phosphate buffer, pH 7. Spectra at t=0 (1), 50 µs (2), 100 µs (3), 200 µs (4), and t=∞ (5) after flash initiation. Insert: Time-dependence of the change of absorbance after flash decay, related to the initial flavin concentration. Reproduced from Ref. 39 by courtesy of the American Chemical Society.

Examples for flavin photosubstrates yielding an irreversible electron transfer by decarboxylation are the amino acids ethylenediaminetetraacetate (EDTA) and nitrilotriacetate (NTA). Using these substrates in a concentration, which effectively quenches the flavin triplet, but not the singlet state, a flavin photobleaching is observed with a quantum yield nearly as high as the flavin triplet formation yield for EDTA (Ref. 41,39) and with some lower yield for NTA (Ref. 39). Quantitative formation of carbon dioxide with respect to reduced flavin has been detected analytically for both reactions (Ref. 42). The primary event in the flavin photobleaching is an electron transfer as observed by flash photolysis for EDTA (Ref. 43, 39) and NTA (Ref. 39). From the quantum yield of photobleaching and the analysis of the reaction products, yielding dihydroflavin and the oxidized substrate products glyoxylate for EDTA and formaldehyde for NTA (Ref. 42), it follows that two electron equivalents are transferred stoichiometrically from the photosubstrate in the overall reaction. From this the question arises, as to how the second electron equivalent is removed from the substrate radical and given to the flavin redox system.

The flavin/EDTA photosystem. The flash photolysis of an aqueous flavin solution in the presence of EDTA (phosphate buffer, pH 7) yields the spectra reproduced in Fig. 3. At the end of the flash the neutral flavosemiquinone is obtained as demonstrated by its absorption at 560 nm, which then decays in a biphasic mode. The first phase is a dismutation reaction as is concluded from the following observation. Within the first 200 µs after the

flash 68 % of the flavosemiquinones with respect to total flavin have decayed. Within the same time range a bleaching of 33 % of the initial absorbance occurred at 483 nm, the isosbestic point between the flavosemiquinone and the flavoquinone. This bleaching, due to flavohydroquinone formation, is about one half of the value of the radical decay. As expected from the dismutation mechanism a re-formation of the flavoquinone absorbance at 440 nm was observed.

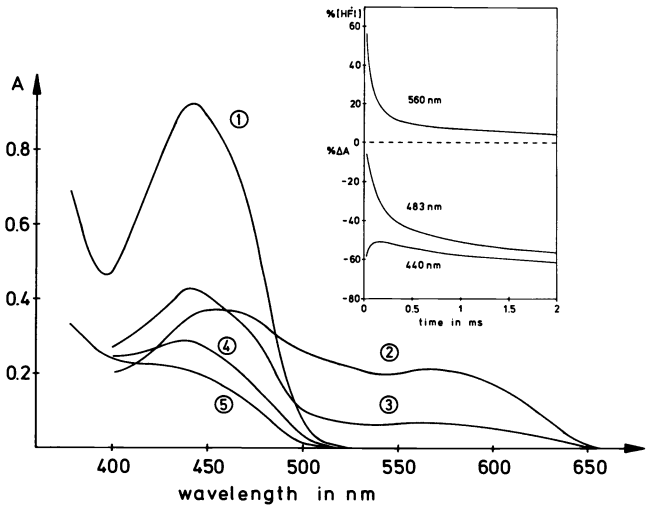



Fig. 3. The photoreduction of 3-methyllumiflavin (7.5 μM) by ethylenediaminetetraacetate (0.01 M) in anaerobic phosphate buffer, pH 7. Spectra at t=0 (1), 25 μs (2), 200 μs (3), 10 ms (4), and t=∞ (5) after flash initiation. Insert: Time-dependence of the change of absorbance after flash decay, related to the initial flavin concentration. Reproduced from Ref. 39 by courtesy of the American Chemical Society.

During the second, slower phase of the flavosemiquinone decay a secondary bleaching of the flavin absorbance occurs in addition to the primary bleaching, brought about by the flash (Fig. 3). Considering the reactions required thus far to explain the experimental results, Eqs. 13-15, we conclude that the decarboxylated substrate radical $\dot{R}$ is the only potent electron donor to reduce flavin in the dark. The reaction yields the flavosemiquinone and the oxidized substrate (Eq. 16).

$$^3Fl_{ox}^* + R\text{-}COO^- \xrightarrow{+H^+} \dot{H}Fl + {}^+\dot{R}\text{-}COO^- \qquad (13)$$

$$2\ \dot{H}Fl \xrightarrow{-H^+} Fl_{ox} + HFl_{red}^- \qquad (14)$$

$${}^+\dot{R}\text{-}COO^- \longrightarrow \dot{R} + CO_2 \qquad (15)$$

$$Fl_{ox} + \dot{R} \xrightarrow{+H^+} \dot{H}Fl + R^+ \qquad (16)$$

From Eqs. 14-16 the following kinetic relations are derived:

$$-\,d[\dot{H}Fl]/dt = 2k_{dism}\,[\dot{H}Fl]^2 - k_R[Fl_{ox}][\dot{R}] \qquad (17)$$

$$-\,d[Fl_{ox}]/dt = k_R[Fl_{ox}][\dot{R}] \qquad (18)$$

As can be seen from Eq. 17 the decay of the flavosemiquinone by dismutation is counteracted by its re-formation, the latter being the more pronounced the higher the initial flavin concentration. Therefore, the biphasic decay mode is more clearly seen at higher flavin concentrations, and that is exactly what is observed experimentally (Ref. 39).
It follows from Eq. 18 that the secondary flavin bleaching reaction should be faster if the concentration of one of the two reaction partners is increased. Indeed, this prediction was confirmed by an experiment in which we increased the initial flavin concentration while keeping the substrate radical concentration constant. The latter was done by lowering the flash intensity to compensate for the increase of light absorption due to the change of optical density of the solution. This was checked by determination of the flavosemiquinone yield, which according to Eqs. 13 and 15 is equal to the yield of the decarboxylated substrate radical. The flavin bleaching was much faster at higher flavin concentration, whereas neither the total amount of bleaching nor the rate constant changed (Ref. 39).

The flavin/NTA photosystem. The flash photolysis of an aqueous flavin solution in presence of NTA (pH 7) leads to the same flavosemiquinone chromophore as observed in the former photosystem (Fig. 4). However, the flavosemiquinone decay is now monophasic in a second order reaction. The amount of flavosemiquinone decaying within the first 200 μs after flash initiation is found to be 42 % of total flavin. At 483 nm, nearly the same percentage of total flavin, namely 38 %, is found to be bleached within the same time range. This demonstrates that one bleached product molecule is formed directly from one flavosemiquinone in the radical decay process.

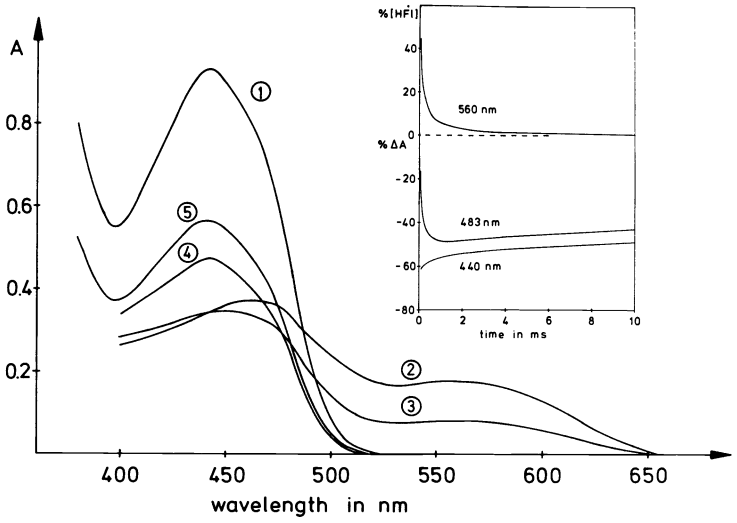



Fig. 4. The photoreduction of 3-methyllumiflavin (7.5 μM) by nitrilotriacetate (0.2 M) in anaerobic aqueous solution, pH 7. Spectra at t=0 (1), 50 μs (2), 200 μs (3), 20 ms (4), and t=∞ (5) after flash initiation. Insert: Time-dependence of the change of absorbance after flash decay, related to the initial flavin concentration. Reproduced from Ref. 39 by courtesy of the American Chemical Society.

If an electron transfer from the substrate radical to the flavosemiquinone is assumed, the final reaction products should be formed and the reaction should cease. However, this is not true for the photosystem under investigation. Instead, the experiments show a re-formation of the flavin absorbance in a time range of seconds, i.e. after all of the flavosemiquinones have decayed.
This finding is explainable by the assumption of transient species that absorb less between 400 nm and 500 nm than the oxidized flavin. These species should be formed by radical combination (Eq. 19) and should decay heterolytically to yield either the flavohydroquinone (Eq. 20) or the flavoquinone

(Eq. 21) depending upon the position of substrate radical addition to the flavosemiquinone molecule. Such covalently bound flavin-substrate-adducts are well known in flavin photochemistry (Ref. 44) and in biochemistry (Ref. 45).

$$\dot{HFl} + \dot{R} \longrightarrow R\text{-}HFl_{red} \quad (19)$$

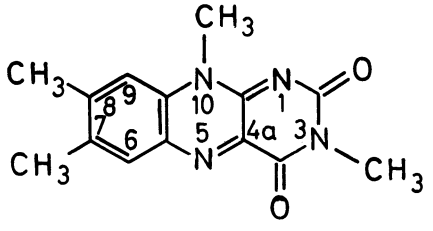
$$R\text{-}HFl_{red} \longrightarrow R_{ox}^{+} + HFl_{red}^{-} \quad (20)$$

$$R\text{-}HFl_{red} \longrightarrow RH + Fl_{ox} \quad (21)$$

Indeed, an absorbance that decayed concomitantly to the flavin re-formation could be monitored in the region of 300 nm. These adducts, which have an absorption spectrum similar to that of reduced flavin are known to absorb more at this wavelength than the oxidized flavin (Ref. 39). It was concluded from the analysis of the course of decay of the absorbance, that three different adduct-species must exist, which is in agreement with the number of types of flavin-adducts, described by Hemmerich et al. (46).

The mechanism of the flavin photoreduction by EDTA and NTA can be understood in the light of the present results. The photoinduced electron transfer becomes irreversible by the decarboxylation process in the substrate radical. The fate of the irreversible oxidized substrate radical is now confined to a second oxidation step. Dismutation is disfavored for EDTA and NTA due to the lack of $\beta$-$CH_2$ groups, which prevent enamine formation. From the product pattern in the overall EDTA reaction, which yields glyoxylate in contrast to formaldehyde in the NTA reaction, an internal 1,6-hydrogen shift is deduced in the EDTA radical fragment; this leads to a more stable secondary radical (Fig. 5).
Such a hydrogen shift is not possible in the NTA radical and can be prevented in the EDTA radical by chelating EDTA with metal cations. In the latter case the product pattern of the flavin/NTA photoreaction is obtained (Ref. 42). Whereas the NTA radical adds to the flavosemiquinone to yield a flavin-substrate-adduct, which in turn decays by heterolytic bond splitting, the secondary EDTA radical is sterically too hindered to undergo a fast addition reaction and will be too stable to transfer its unpaired electron with a rate which can compete with the flavosemiquinone dismutation. Therefore, electron transfer to flavin, which is then the only oxidant present after longer times, is the only decay mode for the EDTA radical fragment.

Very recently, a stable flavin-substrate-adduct could be isolated which is formed by radical combination in the flavin photoreduction by N-allylthiourea (Ref. 47). Quantitation of intermediates and reaction products showed that the radical combination is competitive to electron back transfer. Obviously, in this case, the deprotonation in the N-allylthiourea radical cation is rather slow or ineffective in decreasing the reduction potential of the radical state compared to the fast decarboxylation process in the mechanism of flavin-sensitized photooxidation of amino acids.




3-methyllumiflavin

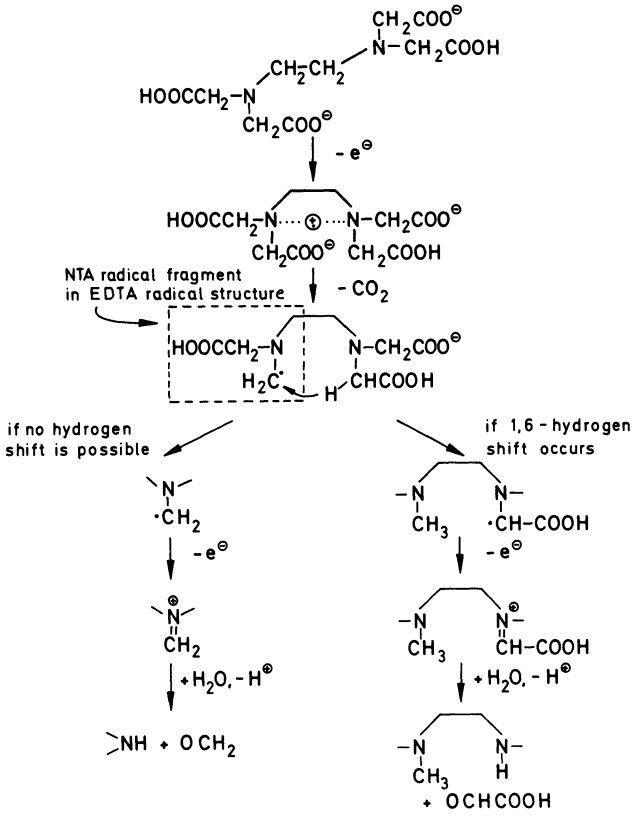



Fig. 5. The reaction pathways of flavin-sensitized photooxidation of ethylenediaminetetraacetate and nitrilotriacetate. Reproduced from Ref. 42 by courtesy of Pergamon Press Ltd..

## THE TWO-ELECTRON TRANSFER

Nucleophilic attack of aqueous and methanolic solvent molecules on the protonated or alkylated flavoquinonium cation in the photoexcited singlet state has been observed by Schöllnhammer and Hemmerich (48). Said attack yields 6- and 9-hydroxyflavins and 9-methoxyflavins, respectively, in aerobic solution. This was another proof for the ability of photoexcited quaternary salts of N-heteroaromatic compounds to undergo addition reactions as reviewed by Whitten (49). Flash photolysis investigations of Düren et al. (50) of the photohydration of lumichrome, an alloxazine dye, in acidic solution demonstrated the anticipated heterolytic addition mechanism in the photoexcited singlet state. Although no true redox change occurs by the addition of the electronegative oxygen of the water or alcohol molecule to the carbon skeleton of the photoexcited flavin cation (Eq. 22), in view of the π-electron system of the flavin, an electron pair is accepted. This interrupts the electron conjugation and leads to the dihydroflavin chromophore. The photoaddition product obtained is easily oxidized in presence of oxygen to yield the hydroxy- and methoxy-substituted compounds, mentioned above, which represent oxygenated flavins.

$$^{1}HFl_{ox}^{+*} + R\text{-}OH \longrightarrow RO\text{-}Fl_{red}H + H^{+} \quad ; \quad R = H, \text{ alkyl} \qquad (22)$$

Extending the concept of Brønsted's acid-base reactions in the photoexcited state, which is closely related to the names of Förster and Weller (Ref. 51), the kind of photoreactions described has to be considered as a neutraliza-

tion reaction of a hard Lewis acid (photoexcited, protonated flavin singlet) with a hard Lewis base (water or alcohol) and as redox reaction only in terms of flavin spectrophotometry.
In the following section we will present experimental evidence that the neutral, photoexcited flavin triplet is a soft Lewis acid, which accepts easily polarizable electron pairs from the complexed hydride ion and the free cyanide ion to yield, in an one-step process, a two electron reduction product in terms of redox stoichiometry (Eq. 23).

$$^3Fl_{ox}^* + R^- \longrightarrow R\text{-}Fl_{red}^- \quad ; \quad R = CN,\ H\ (\text{from } BH_4^-) \qquad (23)$$

The photohydrogenation of flavin

During their study of new flavohydroquinone structures Müller and colleagues (52) observed that the slow flavin reduction by borohydride is increased strongly by exposing the reaction mixture to light. Based on our knowledge of the reduction potentials of photoexcited states of flavin, we concluded that the electron affinity will not be high enough to induce the transfer of a single electron from the borohydride ion. Therefore, we assumed that the borohydride ion would be a potential model for a two-electron transferring flavin photosubstrate. This was proven experimentally and will be discussed by means of Fig. 6. The figure is obtained from an experiment in which an oxygen-free, aqueous flavin solution (pH 7) is flashed in presence of sodium cyanoborohydride (Ref. 53,54). The concentration of the borohydride was chosen so as to quench the flavin triplet but not the photoexcited singlet state. The concentration of flavin and flavosemiquinone are shown in the figure as a function of time after the flash. The flavohydroquinone concentration is obtained by subtracting the flavin and flavosemiquinone concentration from the total flavin concentration and is represented by the hatched area.

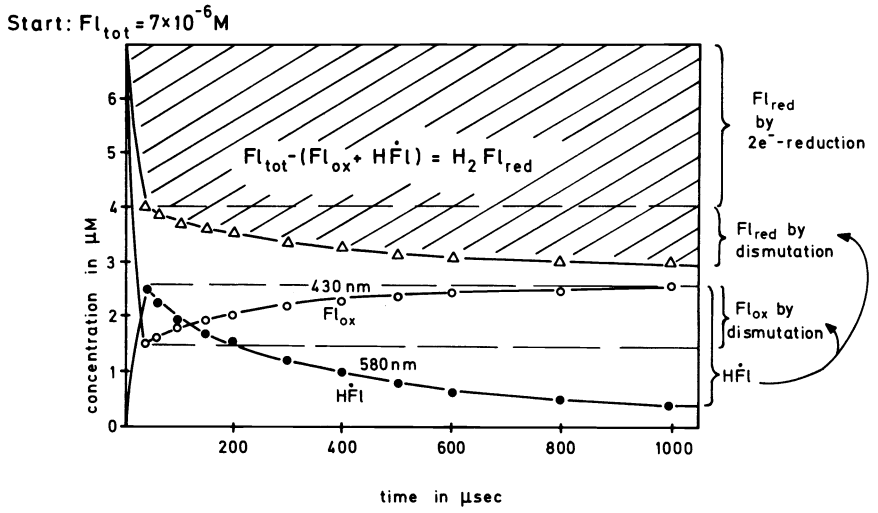



Fig. 6. The time-dependence of the concentration of flavin redox states after the flash in the photoreduction of 3-methyllumiflavin (7 µM) by sodium cyanoborohydride (17 mM) in anaerobic phosphate buffer, pH 7. Reproduced from Ref. 53 by courtesy of the Federation of European Biochemical Societies.

The figure shows clearly, that, just after the flash, a large part of the flavin triplet molecules are converted directly to the flavohydroquinone (Eq. 24). The origin of the flavosemiquinone, the one-electron transfer product, is also explained by this mechanism: unfortunately, a borohydride concentration cannot be used that is high enough to quench all the triplet molecules, because the flavin photoexcited singlet state should not be quenched to a noticeable extent. Therefore, the remaining flavin triplet

molecules will undergo a fast reaction with the product of the hydride transfer, the flavohydroquinone. This photocomproportionation (Eq. 25) is reversible in the dark (Eq. 26), and this is shown in Fig. 6, in which two flavosemiquinone molecules are converted, during the time after the flash, in one flavoquinone and one flavohydroquinone molecule.

$$^3Fl_{ox}^* + BH_3CN^- \longrightarrow HFl_{red}^- + 1/2\ (BH_2CN)_2 \quad (24)$$

$$^3Fl_{ox}^* + HFl_{red}^- \xrightarrow{+H^+} 2\ \dot{H}Fl \quad (25)$$

$$2\ \dot{H}Fl \xrightarrow{-H^+} Fl_{ox} + HFl_{red}^- \quad (26)$$

The product quenching mechanism was confirmed by flashing a solution containing flavohydroquinone in a concentration comparable to the former reaction yield in addition to flavin and cyanoborohydride. The result was compared to that of the solution without additional flavohydroquinone. As expected, the comparison proves that the additional flavohydroquinone causes an increase of the flavosemiquinone and a decrease of the yield of permanent bleaching. In addition, the competitive behavior is further demonstrated by a series of experiments, in which the flavin triplet quenching is increased by increasing the borohydride concentration. A non-linear correlation between the flavohydroquinone and the flavosemiquinone results, the yield of the photohydrogenation product increases much faster than the yield of the photocomproportionation product (Ref. 53).
Using cyanoborodeuteride as the reducing agent and analyzing the products of the flavin photoreduction by mass and NMR spectroscopy, the site of hydride fixation was revealed to be the 9- and 6-position of the flavin chromophore (Ref. 53).

The photocyanation of flavin

With the hydride ion as the two-electron carrier a light-induced heteropolar C-H bond formation at the flavin nucleus resulted. We found the cyanide ion to be the most suitable nucleophilic flavin photosubstrate to obtain a C-C bond formation (Ref. 55).
Preparative and analytic work showed that the cyanide ion adds to the photoexcited flavin triplet (like the hydride ion) in position 9 and 6. The 9- and 6-cyanoflavohydroquinones thus obtained are easily oxidized with oxygen to the cyano-substituted flavins.

When we flash flavin in presence of 0.2 molar sodium cyanide in an anaerobic, aqueous solution of pH 11, the total flavin triplet is quenched, while the flavin fluorescence is diminished only for 14 %, and we observe a permanent bleaching at 440 nm, which represents a reaction yield of 80 % per initial flavin (Fig. 7). The intermediate absorbance built up by the flash shows a biphasic change. After the initial bleaching during the flash, an increase of absorbance is observed, which reaches a maximum at about 500 µs and then decays very slowly, relative to the first phase. Monitoring the time course of the absorbance at the isosbestic point between the flavosemiquinone anion and the flavoquinone at 485 nm (Ref. 55), a large bleaching is detected just after the flash and the direct conversion of the flavin triplet to the two-electron reduction product is derived by extrapolating the curve to zero time.
In addition, by calculating the theoretical course of absorbance of the flavosemiquinone anion decay (which has to occur in a hypothetical radical mechanism of the photocyanation reaction), it was shown that the change of absorbance actually observed is far away from the region which is admissible for the radical reaction. Instead, the biphasic change of absorbance observed is in agreement with the result of preparative photochemistry that shows two isomeric cyanide addition products. The change of absorbance reflects the two relaxation processes of the species involved in the required 6,5- and 9,5-prototropic shift.

Determination of reaction rate constants shows, that the cyanoborohydride and the cyanide ion are equally effective in attacking the flavin triplet, but differ in quenching the photoexcited flavin singlet:

| | $k_q$-singlet | $k_q$-triplet |
|---|---|---|
| $BH_3CN^-$ | $1.2\times10^9$ $M^{-1}s^{-1}$ | $4\times10^6$ $M^{-1}s^{-1}$ |
| $CN^-$ | $1.5\times10^8$ $M^{-1}s^{-1}$ | $6\times10^6$ $M^{-1}s^{-1}$ |

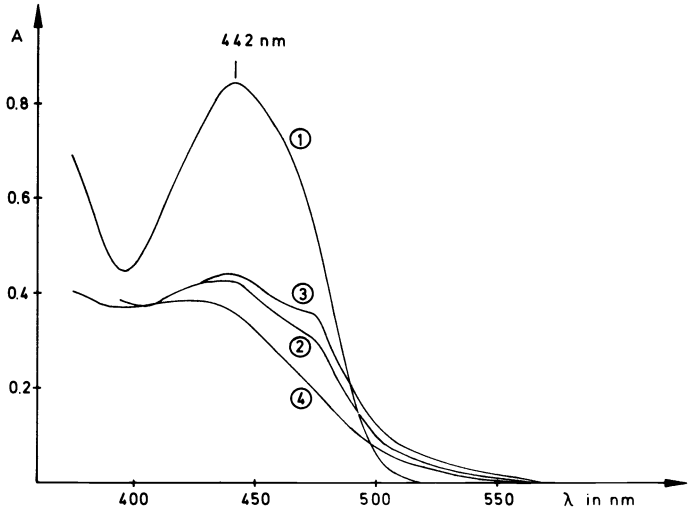



Fig. 7. The photoreduction of 3-methyllumiflavin (7 μM) by sodium cyanide (0.2 M) in anaerobic, aqueous solution, pH 11. Spectra at t=0 (1), 50 μs (2), 500 μs (3), and t=∞ (4) after flash initiation. Reproduced from Ref. 55 by courtesy of Pergamon Press Ltd..

This may be explained with the concept that the neutral photoexcited flavin singlet is also a hard Lewis acid which, according to the Pearson principle, prefers the harder hydride ion from borohydride over the soft cyanide ion as Lewis base.
The photohydrogenation and photocyanation of flavin is thus very similar to the heteropolar nucleophilic aromatic photosubstitution reaction, investigated by Havinga, Cornelisse and collaborators (56,57). The flavin photoreaction differs in the way by which the aromaticity is restored. In the aromatic photosubstitution the two-electron package, brought about with the attacking nuclophile, is carried away by the leaving group. Whereas, in case of the flavin photoreduction, the primarily formed complex is stabilized by a prototropic shift which restores the aromaticity in the benzene nucleus by limiting the two-electron package to the pteridine part of the flavin molecule. Under aerobic conditions oxygen is then the external electron acceptor in the re-oxidation process, leading to substituted flavoquinones.

## CONCLUSION

Competitive pathways of photoreduction have been observed with the same dye chromophore. Substrates with one-electron oxidation potentials lower than 2.0 V, 1.8 V and 1.55 V vs. s.c.e. induce one-electron transfer processes with a rate constant larger than $10^6$ $M^{-1}s^{-1}$ in the photoexcited flavin singlet, protonated triplet and neutral triplet state. Lewis bases with higher oxidation potentials are two-electron photosubstrates, which transfer their electron pair to the photoexcited, protonated flavin singlet, if they are hard, and to the photoexcited flavin triplet, if they are soft. The increase in reactivity of one-electron transfer is due to the promotion of an electron into a higher molecular orbital by the absorption of light and, therefore, the reduction potential of the dye is increased by the ex-

citation energy, as shown by Leonhardt and Weller (Ref. 3, for a recent discussion cf. Ref. 58). But, if there is a multiplicity change on the reaction pathway, the reactivity of the photoexcited dye becomes a little lower than expected from the above concept. This can be explained by an exciplex as an intermediate and the resulting kinetic considerations. Photoexcitation also increases the electrophilicity of the flavin molecule and changes the position of nucleophilic substrate attack from C-4a and N-5 in the ground state (Ref. 44) to the positions C-6 and C-9 in the photoexcited singlet and triplet state.
Nevertheless, the one-electron transfer pathway is more activated by absorption of light. This is shown by the reaction with the sulfite ion, which is a two-electron substrate in flavin ground state chemistry, yielding a N-5 flavin-substrate-adduct (Ref. 59). However, it is revealed to be an one-electron donor in flavin photochemistry (Ref. 60).

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