Mechanism of Light-Induced Reduction of Biological Redox Centers by Amino Acids. A Flash Photolysis Study of Flavin Photoreduction by Ethylenediaminetetraacetate and Nitrilotriacetate†

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ABSTRACT: The mechanism of flavin photoreduction by the amino acids, EDTA, and nitrilotriacetate is shown to be due to light-induced charge separation, which is irreversible in the dark. The irreversibility originates from the decarboxylation of the amino acid radical. This fast process changes the redox properties of the radical and makes a further donation of an electron equivalent possible. In the case of EDTA the electron acceptor of the second electron is flavin, which was left unexcited by the flash or is formed by dismutation from the flavosemiquinone, generated in the primary one-electron transfer process. In contrast to this, a mechanism for the flavin photoreduction by nitrilotriacetate is proposed, in which the decarboxylated nitrilotriacetate radical adds to the flavosemiquinone to yield an alkylated flavohydroquinone. The latter decays to free reduced or oxidized flavin, depending on the position of addition at the flavin chromophore. The difference in reaction mechanism between the nitrite anion, EDTA, and nitrilotriacetate is discussed in terms of differences in molecular structure.

From the early periods of flavin photochemistry, EDTA[1] was used as a photoreductant and now has seen a steadily widening application as donor of redox equivalents in the photochemistry of dyes [see, for example, Morita & Kato (1969), Bonneau et al. (1973), Usui et al. (1980), and Krasna (1980)] and in the study of redox–enzyme mechanisms (Massey et al., 1978; Massey & Hemmerich, 1978).

Originally, Merkel & Nickerson (1954) were surprised by the finding that riboflavin could be photoreduced in EDTA buffer, which they explained by a cleavage of water through the excited flavin chromophore. Obviously, such a reaction is energetically unfeasible with blue light (450 nm). Frisell et al. (1959) corrected this interpretation by evidence that the buffer was the reductant. Thus, it was found that the well-known photodestruction of riboflavin (Warburg & Christian, 1932; Kuhn et al., 1933) could be prevented by the presence of EDTA, since the reduced flavin would be quantitatively reoxidized by O_2.

Holmström (1964) was first to see spectrophotometrically the semiquinoid form of the free flavin in the Fl_{ox}/EDTA photosystem by flash photolysis, while Massey & Palmer (1966) first used the photoirradiation method in the presence of EDTA to convert flavoproteins in the semiquinoid form. Recently, nanosecond laser flash photolysis experiments confirmed the electron transfer to be the primary quenching process of the photoexcited flavin triplet state. This follows from a transient, first observed by Visser et al. (1977), being an intermediate between $^3Fl_{ox}$* and HFl in the EDTA photoreaction. Heelis et al. (1979a) showed that this transient is Fl^-, which decays by protonation to HFl in neutral solution.

Quantitation of the photoreaction showed that the quantum yield of flavin photoreduction by EDTA is nearly as high as the quantum yield of flavin triplet formation (Moore et al., 1977; Heelis et al., 1979b). In addition, product analysis revealed that two electron equivalents are transferred from the EDTA molecule to the flavin in the overall reaction (Enns & Burgess, 1965; Armstrong et al., 1982).

In contrast to the primary process of the photoreaction mechanism, which is now well elucidated, in fact, nothing is known about how the second electron equivalent is transferred. To shed more light on this process a flash photolysis study was undertaken, following spectrophotometrically the fate of ground-state flavin and intermediates in the reaction of flavin-sensitized photooxidation of EDTA and NTA.

Materials and Methods

Chemicals. 3,7,8,10-Tetramethylisoalloxazine (3-methyllumiflavin) was synthesized according to Hemmerich (1964). The substrates EDTA (ethylenediaminetetraacetic acid disodium salt dihydrate) and NTA (nitrilotriacetic acid; titrated with NaOH to yield pH 7) and $NaNO_2$ were analytical grade from Merck or Fluka. Doubly distilled water was used as solvent. The phosphate buffer, pH 7, was 0.05 M (Merck's titrisol buffer). Oxygen was removed by flushing the solutions for at least 30 min with solvent-saturated nitrogen. The gas was purified by passing over "Oxysorb" (Messer Griesheim GmbH), yielding $[O_2] < 0.1$ ppm.

Apparatus. The kinetic flash apparatus has been described by Vogelmann et al. (1976). The input energy of the flash was 600 J. The length of the flash profile at half of its amplitude (fwhm) was about 8 μs. The cuvette (10 cm) was surrounded by a Kodak–Wratten gelatin filter, No. 2A (transmittance 0.1% for wavelengths < 410 nm), to limit the irradiation to the first absorption band of flavin. For the registration of the transients, a cutoff filter (transparent for $\lambda > 520$ nm), an interference filter monochromator (400–700 nm; Oriel Optics Corp.), or special interference filters for wavelengths below 400 nm were mounted in the monitoring light beam in order to prevent further photochemistry.

A Zeiss DMR 10 spectrophotometer was used to record the UV–vis spectra before and after flashing and a Perkin-Elmer

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[1] Abbreviations: Fl_{ox}, HFl, and H_2Fl_{red}, the oxidized, neutral semiquinoid, and fully reduced forms of flavin; $^1Fl_{ox}$* and $^3Fl_{ox}$*, the photoexcited singlet and triplet states of flavin; EDTA, ethylenediaminetetraacetate; NTA, nitrilotriacetate.

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Table I: Photochemical Data

substrate	k_q^S (M^{-1} s^{-1})[a,c]	k_q^T (M^{-1} s^{-1})[b,c]	concn of substrate[d] (M)	quenching (%) of $^1Fl_{ox}$*	quenching (%) of $^3Fl_{ox}$*	yield of HFl (%)[e,f]	yield of permanent Fl_{ox} bleaching (%)[e,g]
NO_2^-	(6.6 ± 0.1) × 10^9	(1.5 ± 0.3) × 10^9	0.003	9	99.1	83 ± 5	0
EDTA	(9.7 ± 0.2) × 10^8	(6.2 ± 0.3) × 10^8	0.01	4	99.4	85 ± 5	84 ± 5
NTA	(9.5 ± 0.4) × 10^7	(3.6 ± 0.7) × 10^6	0.2	9	95	64 ± 4	42 ± 4

[a] Excited singlet quenching rate constant, determined according to $I_0/I = 1 + k_q^S\tau[Q]$, with $\tau = 5.0$ ns (Byrom & Turnbull, 1968; Gordon-Walker et al., 1970). [b] Excited triplet quenching rate constant, determined according to $k_{exp} = k_0 + k_q^T[Q]$. [c] 3-Methyllumiflavin (2×10^{-5} M) in phosphate buffer, pH 7. [d] 3-Methyllumiflavin (7.5×10^{-6} M) in phosphate buffer, pH 7. [e] Per flash in percent total flavin. [f] The absorbance of HFl was monitored at 560 nm and extrapolated to $t = 9$ μs [see Materials and Methods and Goldberg et al. (1981)]; $\epsilon(HFl) = 5000$ M^{-1} cm^{-1} for $\lambda = 560$ nm was used (Land & Swallow, 1969). [g] The change of absorbance was monitored at 442 nm, and the yield was calculated according to $[(A_{t=0} - A_{t=\infty})/[\epsilon(Fl_{ox}) - \epsilon(HFl_{red}\cdot)]d](100/[Fl_{ox}]_{t=0})$ with $\epsilon(Fl_{ox}) - \epsilon(HFl_{red}\cdot) = 12\,300$–$1100$ M^{-1} cm^{-1}.

MPF-3L fluorescence spectrophotometer for fluorescence quenching experiments.

Methods. The transient spectra were obtained in the following way: The change of absorbance after the flash was recorded at least 3 times as a function of time at wavelengths from 400 to 700 nm in 5-nm intervals. The range of noise was $\Delta\Delta A = 0.02$. The transient spectra were now constructed by adding the change of absorbance, ΔA ($t = x$), measured at a given time x from flash initiation, to the absorption spectrum of the solution, initially monitored by means of the Zeiss spectrophotometer.

The radical yield per flash was determined by extrapolation of the second-order decay reaction to the time 9 μs after flash triggering. For this we had the following reason: Since our flash lifetime is not negligibly short with respect to the radical decay time, the end of radical production and the beginning of decay, respectively, will not be limited to a precise point of time. Therefore we determined the time which yields the smallest error, namely, 9 μs, as follows:

Equation 1, which was derived from the integrated rate law

$$\frac{[H\dot{F}l]}{[^3Fl_{ox}^*]_0} = \left([^3Fl_{ox}^*]_0 kt + \frac{[^3Fl_{ox}^*]_0}{[H\dot{F}l]_0} \right)^{-1} \quad (1)$$

of a second-order reaction by multiplication with the excited triplet concentration ($[^3Fl_{ox}^*]_0$), shows that the apparent radical yield ($[H\dot{F}l]/[^3Fl_{ox}^*]_0$), observed at a given time, depends on the initial excited triplet state concentration and the time. According to this equation, the radical yield is independent of the initial excited triplet concentration at the starting point of decay, $t = 0$, and for this time the true radical yield is obtained.

Applying this to our conditions, the most exact point of starting time will be characterized by a minimum of the differences of radical yields, obtained by reactions, which involve different concentrations of excited triplets.

These reactions are experimentally realized by altering the flash input energy. From those experiments the best point of starting time was found to be 9 μs after flash initiation as described by Goldberg et al. (1981).

Results and Discussion

Standardization of Reaction Conditions. For characterization of the reactivity of the substrates, fluorescence and triplet quenching rate constants were determined according to the Stern-Volmer treatment, as listed in Table I. From these data substrate concentration was calculated, which shortens the triplet lifetime to more than 95% but at the same time affects the flavin fluorescence to only less than 10%. This ascertains the confinement of flavin photochemistry to the flavin triplet state (cf. Table I).

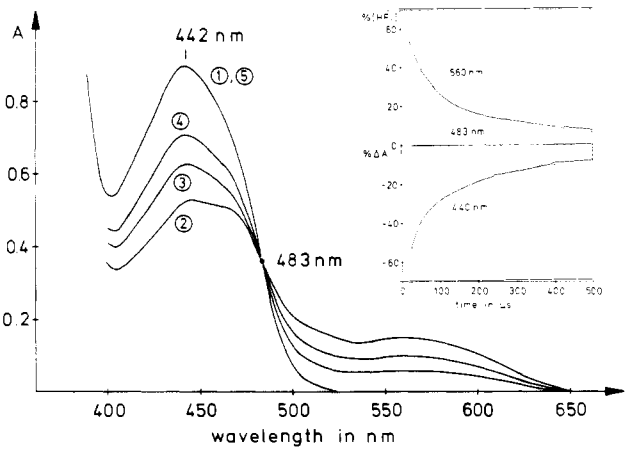


FIGURE 1: Reversible one-electron photoreduction of 3-methyllumiflavin (7.5 μM) by $NaNO_2$ (0.01 M) in anaerobic phosphate buffer, pH 7. Spectra before (1) and after one flash (5). Transient spectra at 50 (2), 100 (3), and 200 μs (4) after flash triggering. (Insert) Time dependence of the change of absorbance after flash decay, related to the initial Fl_{ox} concentration. The decay of HFl (560 nm) is concomitant with the reappearance of Fl_{ox} (440 nm). At 483 nm an isosbestic point exists between HFl and Fl_{ox}.

Nitrite Anion as Calibrating Substrate: The Reversible One-Electron Transfer. When we flash 3-methyllumiflavin, 7.5 μM, in the presence of 3 mM $NaNO_2$ in phosphate buffer, pH 7, we arrive at a yield of neutral flavosemiquinone (Dudley et al., 1964; Land & Swallow, 1969) as high as 83% of total flavin. Further increase of the nitrite concentration will decrease the radical yield, due to the concomitant increase of fluorescence quenching by nitrite, which diminishes the triplet state population on flash excitation. This implies that anion-induced fluorescence quenching does not result in free radical production in agreement with nanosecond laser photolysis experiments of Shizuka et al. (1980).

The HFl radicals obtained decay with a reaction rate constant of 3.4×10^9 M^{-1} s^{-1}, as observed at 560 nm with subsequent reconversion of Fl_{ox}, monitored at 440 nm (Figure 1). The photochemical reaction between flavin and nitrite is therefore revealed as a reversible one-electron transfer reaction (eq 2). Accordingly, an isosbestic point is formed at 483 nm,

$$Fl_{ox} + NO_2^- \underset{-H^+}{\overset{h\nu, +H^+}{\rightleftharpoons}} H\dot{F}l + NO_2 \quad (2)$$

where the absorbance remains constant (Figure 1). Since the molar absorption coefficient of NO_2 at 483 nm is as low as 80 M^{-1} cm^{-1} (Beattie & Vosper, 1961), it can be neglected compared with the Fl_{ox} absorption [$\epsilon(Fl_{ox})$ at 483 nm = 4900 M^{-1} cm^{-1}]. Therefore at this wavelength $\epsilon(Fl_{ox}) = \epsilon(H\dot{F}l)$ is valid.

Table II: Decay of Flavosemiquinone

substrate	flavin[a]	second-order decay constant[b] (M^{-1} s^{-1})	mode of decay
NO_2^- (0.003 M)	3MLf (7.5×10^{-6} M)	$k = 3.4 \times 10^9$	back-donation
EDTA (0.01 M)	3MLf (7.5×10^{-6} M)	$k_1 = 3 \times 10^9, k_2 = 1 \times 10^9$	biphasic decay: phase 1, dismutation;
	3MLf (5×10^{-5} M)	$k_1 = 2 \times 10^9, k_2 = 5 \times 10^8$	phase 2, re-formation of HFl and
	3ALf (7×10^{-6} M)[c]	$k_1 = 1 \times 10^9, k_2 = 7 \times 10^8$	dismutation
NTA (0.2 M)	3MLf (7.5×10^{-6} M)	$k = 2.2 \times 10^9$	radical combination

[a] 3-Methyllumiflavin (3MLf) and lumiflavin 3-acetate (3ALf) in aqueous solution, pH 7. [b] Monitored at 560 nm and using $\epsilon(HFl) = 5000$ M^{-1} cm^{-1} (Land & Swallow, 1969). [c] To compare, $2k_{dism}(3ALf) = 1.2 \times 10^9$ (Hemmerich et al., 1980).

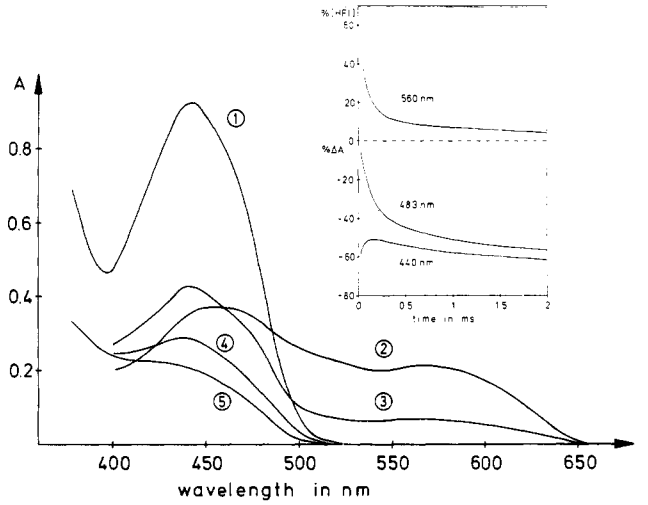


FIGURE 2: Photoreduction of 3-methyllumiflavin (7.5 μM) by EDTA (0.01 M) in anaerobic phosphate buffer, pH 7. Spectra before (1) and after one flash (5). Transient spectra at 25 μs (2), 200 μs (3), and 10 ms (4) after flash triggering. (Insert) Time dependence of the change of absorbance after flash decay, related to the initial Fl_{ox} concentration. At the isosbestic point between HFl and Fl_{ox} (483 nm), a fast bleaching occurs, which is concomitant with HFl decay (560 nm) and partial Fl_{ox} re-formation (440 nm). In the millisecond time range, an additional bleaching of Fl_{ox} (440 nm) is observed, simultaneously with a small absorption of HFl (560 nm).

EDTA as Substrate: The Irreversible One-Electron Transfer. When we flash 3-methyllumiflavin, 7.5 μM, in the presence of 10 mM EDTA in phosphate buffer, pH 7, the same radical chromophore, as shown in Figure 2, is generated per flash in nearly the same yield per total flavin (Table I). However, the fate of the flavosemiquinone generated with EDTA differs greatly from what is observed in the presence of nitrite: While the nitrite-induced HFl decays in a pure second-order reaction, we obtain for the EDTA-induced HFl a biphasic decay, which is the more pronounced the higher the initial Fl_{ox} concentration (Table II).

At the maximum absorption of Fl_{ox}, 440 nm, we observe at the same time two phases of transient absorbance after the initial bleaching produced by the flash: The first phase is an increase of absorbance and the second phase an additional bleaching at a time range when the initially formed HFl has already largely decayed. Also at the isosbestic point of 483 nm, appearing between Fl_{ox} and HFl in the nitrite reaction, we observe in the EDTA reaction a biphasic change of absorbance: a first fast change, which accompanies the small back-formation of absorbance at 440 nm, and a subsequent slow change, which comes along with the slow bleaching phase at 440 nm and the slow decay of HFl absorbance at 560 nm (Figure 2). The extrapolation of the fast bleaching phase at 483 nm to the end of the flash leads back to the starting absorbance of the solution before the flash. This means that all the flavin, which is excited to the triplet state, is subsequently converted to the flavosemiquinone HFl in accordance with the high semiquinone reaction yield. These facts are definite proof that the HFl formed in the EDTA reaction is an intermediate in the bleaching reaction and that no back-donation of electrons from HFl to a substrate product takes place.

Considering the stoichiometry of this reaction, it follows that for each flavin triplet one radical molecule HFl is generated. Furthermore, from the comparison of the HFl yield and the yield of permanent Fl_{ox} bleaching per flash (Table I)—which is equivalent to the HFl_{red}^- reaction yield (Armstrong et al., 1982)—it follows that one semiquinone equivalent is converted to one flavohydroquinone equivalent. The overall reaction is, therefore, shown to be a transfer of two electron equivalents, running through the semiquinone state, in agreement with earlier literature data (see references in the introduction).

For an explanation of the stoichiometry, a mechanism, postulated most frequently in the literature (Nathanson et al., 1967; Kurtin et al., 1967; Weatherby & Carr, 1970; Heelis et al., 1979b), would be that the secondary reaction after the primary one-electron transfer consists of a second one-electron transfer from the EDTA radical to HFl. This mechanism, however, cannot explain the biphasic decay of the radical nor the secondary bleaching of Fl_{ox}, as seen in Figure 2, which does occur at a time when the primary HFl molecules have practically disappeared. Instead, the following reaction scheme can be reconciled with the experimental data:

By comparison of the dismutation constant, obtained by Hemmerich et al. (1980), with the rate constant for our fast HFl decay (cf. lumiflavin 3-acetate, Table II), we are forced to assume that in the EDTA case we also deal with radical decay by dismutation. Therefore the first part of the reaction should read

$$^3Fl_{ox}^* + R\text{-}COO^- \xrightarrow{+H^+} H\dot{F}l + {}^+\dot{R}\text{-}COO^- \quad (3)$$

$$2H\dot{F}l \xrightarrow{-H^+} Fl_{ox} + HFl_{red}^- \quad (4)$$

Reaction 4 also explains the increase of the 440-nm absorption after the initial bleaching by the flash (Figure 2), since $\epsilon(Fl_{ox}) + \epsilon(HFl_{red}^-)$ is greater than $2\epsilon(H\dot{F}l)$ at this wavelength. Continuing, we have to assume that the substrate radical ${}^+\dot{R}\text{-}COO^-$ which is generated will rapidly and quantitatively release CO_2, since this process decreases the reduction potential of the substrate radical by elimination of the positive charge in the amine skeleton. This renders the photoinduced charge separation irreversible. At the same time, the oxidation potential is decreased for the same reason, and a strongly reductive radical $\dot{R}$ is obtained. The latter radical, in turn, will transfer its unpaired electron to the next best oxidant in solution, namely, a ground state molecule Fl_{ox}, yielding a second radical HFl and a residual substrate cation R^+ (eq 6). The

$${}^+\dot{R}\text{-}COO^- \rightarrow \dot{R} + CO_2 \quad (5)$$

$$Fl_{ox} + \dot{R} \xrightarrow{+H^+} H\dot{F}l + R^+ \quad (6)$$

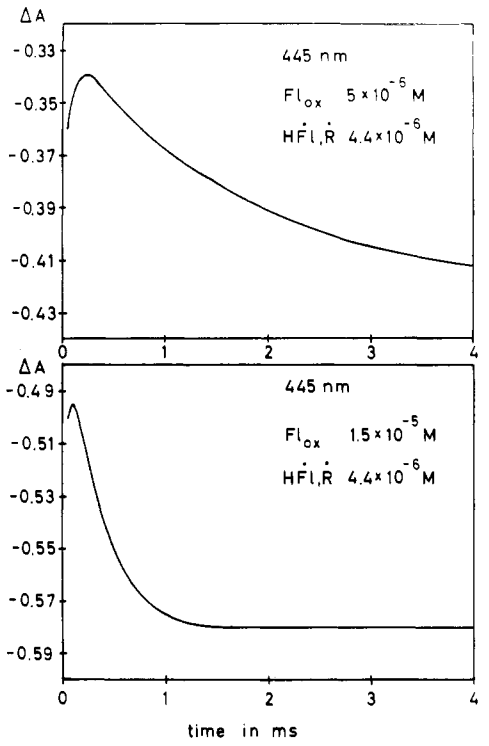


FIGURE 3: Rate of the secondary bleaching reaction in dependence of initial Fl_{ox} concentration in the photoreduction of Fl_{ox} by EDTA. Conditions: EDTA (0.01 M) in aqueous solution, pH 6.5. The flash input energy was diminished from 661 to 180 J to obtain the same initial radical yield, monitored at 560 nm ($H\dot{F}l$), in spite of the change of the absorbance of the solution, which is due to the increase of the Fl_{ox} concentration from 5 (upper part) to 15 μM (lower part).

resulting cation is transparent and disappears subsequently by hydrolysis to yield the reaction product glyoxylate as described by Enns & Burgess (1965) and Armstrong et al. (1982). From eq 5 and 6 the following kinetic relations are derived, applying to the secondary bleaching reaction. In these relations, k_R is the rate constant of reaction 6 and k_{dism} the rate constant of $H\dot{F}l$ dismutation:

$$-d[H\dot{F}l]/dt = 2k_{dism}[H\dot{F}l]^2 - k_R[Fl_{ox}][\dot{R}] \quad (7)$$

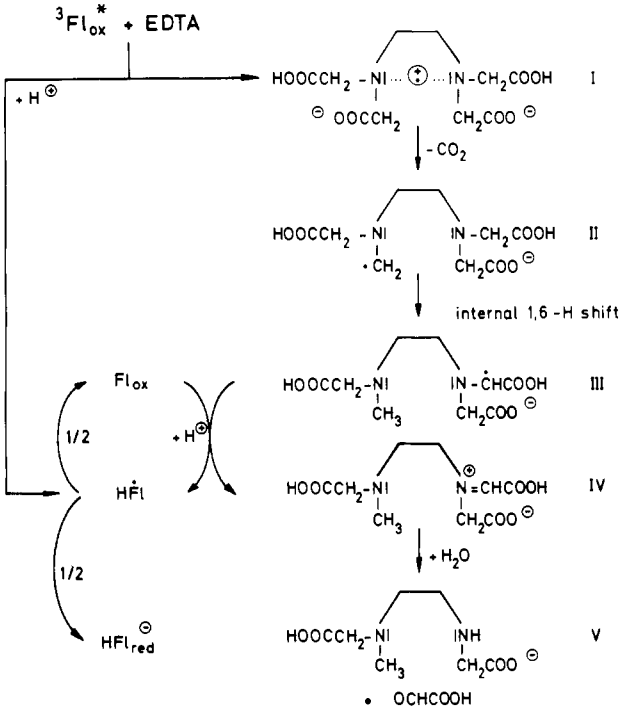
$$-d[Fl_{ox}]/dt = k_R[Fl_{ox}][\dot{R}] \quad (8)$$

As can now be seen from eq 7, the decay of $H\dot{F}l$ by dismutation is superimposed on reformation of $H\dot{F}l$ according to eq 6, and this latter reaction will be the more pronounced, with the higher value of initial Fl_{ox} concentration being chosen. This agrees exactly with what is observed experimentally.

In order to corroborate this evidence, we started another set of experiments, based on eq 8, which states that the secondary Fl_{ox} bleaching reaction will be accelerated if either the Fl_{ox} or the $\dot{R}$ concentration is increased.

So that the effect of increasing the Fl_{ox} concentration could be tested, the substrate radical yield (which according to eq 3 and 5 is equal to the initial $H\dot{F}l$ reaction yield) was kept constant by lowering the flash input energy. This was done in an appropriate manner, to obtain a constant triplet yield, in spite of the change in optical density of the solution. As can be seen in Figure 3 from the comparison of two experiments, differing only in the initial Fl_{ox} concentration, the rate of bleaching is much faster at the higher Fl_{ox} concentration, though the total amount of bleaching does not differ. Furthermore, in both cases the same rate constant $k_R = 2.3 \times 10^8$ M^{-1} s^{-1} is obtained by using $k = (\tau_{1/2}C_0)^{-1}$. This is a final proof of the proposed reaction mechanism, which is outlined in Scheme I.

Scheme I: Mechanism of Flavin Photoreduction by EDTA



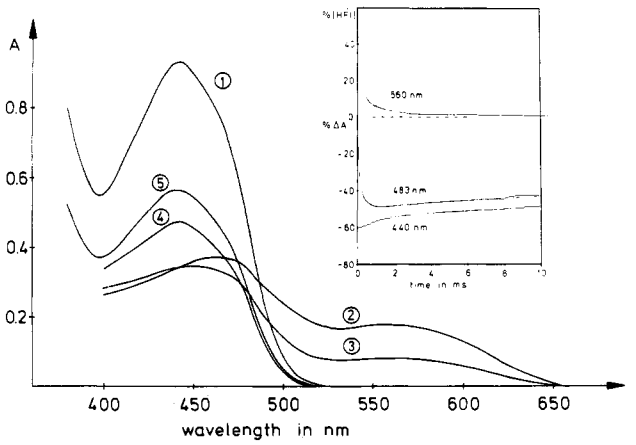


FIGURE 4: Photoreduction of 3-methyllumiflavin (7.5 μM) by NTA (0.2 M) in anerobic aqueous solution, pH 7. Spectra before (1) and after one flash (5). Transient spectra at 50 μs (2), 200 μs (3), and 20 ms (4) after flash triggering. (Insert) Time dependence of the change of absorbance after flash decay, related to the initial Fl_{ox} concentration. At the isosbestic point between $H\dot{F}l$ and Fl_{ox} (483 nm), a bleaching occurs, concomitant with the decay of $H\dot{F}l$ (560 nm). In the millisecond time range, a partial re-formation of Fl_{ox} (440 and 483 nm) is observed.

Very recently, a rate constant of 5×10^8 M^{-1} s^{-1} was obtained by Salet et al. (1981) for the formation of $F\dot{M}NH$ by pulse radiolysis of a N_2O-saturated FMN solution, containing EDTA. They assumed that under their conditions practically all OH radicals are scavenged by EDTA and the FMN reduction must occur via secondary radical attack arising from an EDTA oxidation product. Obviously, their value corresponds to our rate constant of secondary Fl_{ox} bleaching, and an excellent agreement is obtained with respect to the different experimental conditions.

NTA as Substrate: Radical Combination. When we flash 3-methyllumiflavin, 7.5 μM, in the presence of 0.2 M NTA in phosphate buffer, pH 7, the neutral flavosemiquinone is also obtained as shown in Figure 4, but in a somewhat lower yield. Like in the Fl_{ox}/EDTA photosystem, the extrapolation of the signal, monitored at 483 nm, to the end of the flash leads back

to the starting absorbance of the solution before the flash. Therefore, 100% one-electron transfer is revealed as the primary process in the bleaching reaction (eq 3). Since with NTA quantitative release of CO_2 was also found (Armstrong et al., 1982), eq 5 will also be valid.

As outlined above, EDTA-induced HFl decays by dismutation, resulting in 50% bleaching. This follows from experiments which are similar to those shown in the insert of Figure 2 but which were carried out with higher time resolution. In these experiments the HFl decays over the time range of 200 μs from 85% of total flavin to 17%, with the difference being 68%. Within this time range at 483 nm, the isosbestic point between Fl_{ox} and HFl, bleaching of 33% of the total flavin occurs. This is about half of the radical value.

In contrast to this, the NTA-induced HFl decays by 100% bleaching: When the corresponding experiments are done with NTA, the amount of HFl, monitored at 560 nm, which decays within the first 200 μs, is found to be 42% of the 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 proves the stoichiometry one HFl = one bleached product, in the process of primary radical decay.

This clearly demonstrates that the HFl, which is formed in the Fl_{ox}/NTA photoreaction, accepts a second electron equivalent, which will be expressed either by a second transfer of a single electron (or hydrogen atom) from the decarboxylated substrate radical Ṙ or by a recombination of flavin radical and substrate radical:

$$HFl + \dot{R} \rightarrow HFl_{red}^- + R^+ \quad (9)$$

$$HFl + \dot{R} \rightarrow R\text{-}HFl_{red} \quad (10)$$

Both reactions agree with the second-order rate law obtained from the change of absorbance at 560 nm (Table II). The following arguments have to be discussed to decide between the two alternative reaction modes: If the HFl decay consists only in the transfer of a further electron (or hydrogen atom), the Fl_{ox} bleaching reaction should come to a halt when the HFl molecules are decayed, since under anaerobic conditions the stable product HFl_{red}^- is obtained. Surprisingly, we find in the Fl_{ox}/NTA photoreaction a slow re-formation of Fl_{ox}, as shown in Figure 4. The amount is 22% of total flavin, as calculated from Table I. The flash promotes 64% of total flavin in the HFl form, which in turn decays to yield 42% HFl_{red}^-; the difference, therefore, accounts for the reconverted Fl_{ox}.

From the time lag between HFl decay and Fl_{ox} re-formation, the existence of a transient species is postulated, which absorbs less than Fl_{ox} at 440 nm. These qualities are found in unstable substituted dihydroflavins, as described by Hemmerich (1976) and Knappe (1977). Furthermore, for an explanation of the experimental results, the transient species is forced to decay partially by heterolytic bond splitting, yielding Fl_{ox} and RH (eq 11). Another mechanism, which consists of a partial

$$R\text{-}HFl_{red} \rightarrow RH + Fl_{ox} \quad (11)$$

reoxidation of HFl_{red}^-, seems unreasonable to us, because of the lack of an appropriate oxidant under anaerobic conditions. Conceding that the decarboxylated NTA radical may have oxidative qualities, the reductive properties of the same radical should be strong enough to exclude such a long lifetime, as required from the experimental results.

In order to check for the radical combination mechanism, we have searched for the absorbance of this species. The result is shown in Figure 5, in which the change of absorbance after the flash at different wavelengths is presented.

Indeed, at 310 nm, a wavelength which lies between the

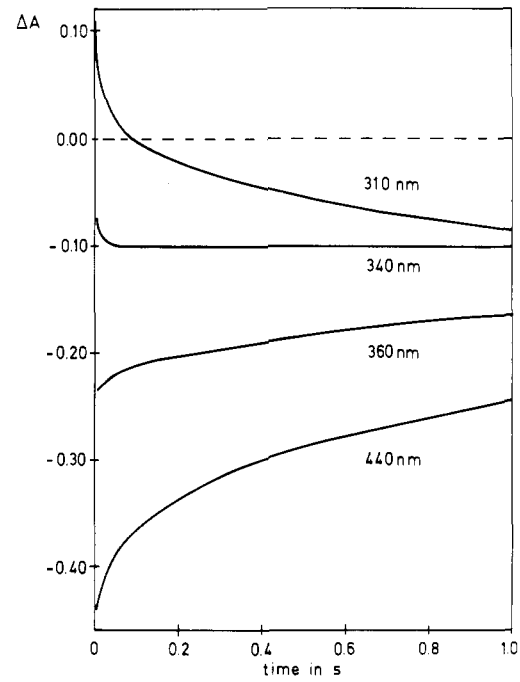


FIGURE 5: Time dependence of the change of absorbance in the photoreduction of Fl_{ox} by NTA in the second time range. The same conditions as in Figure 4 are applied. At 310 nm an absorption occurs, which decays concomitantly with the re-formation of Fl_{ox} (440 nm). The difference between the changes of absorbance at 340 and 360 nm proves that two decaying species exist, one of which has an isosbestic point with Fl_{ox} at 340 nm (narrow band interference filters have been used to prevent bleaching by the monitoring light beam).

third and second absorption band of Fl_{ox} and at which reduced flavins should absorb more than oxidized flavin (Walker et al., 1970), we observe an absorption which appears during the HFl decay (not shown in Figure 5) and decays in the time range of seconds, concomitantly with the increase of the 440 nm absorbance (Figure 5). Furthermore, the results show that these are biphasic processes. This is most clearly seen from the 340- and 360-nm curves in Figure 5, from which it is deduced that two species are involved, a faster decaying one with an isosbestic point with Fl_{ox} between 360 and 340 nm (because of the opposite slopes at 360 and 340 nm in the first 50 ms) and a much slower decaying one with an isosbestic point with Fl_{ox} at 340 nm.

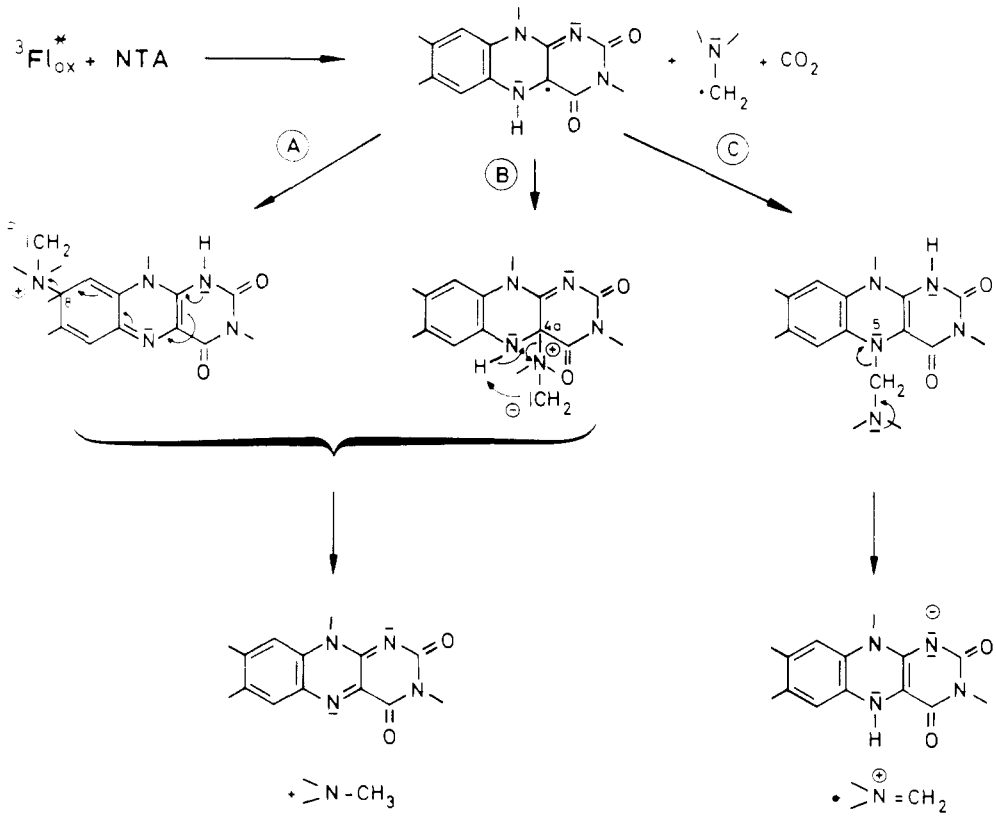
For an explanation of all these facts, a reaction mechanism is suggested as outlined in Scheme II: In analogy with reductive photoalkylation of the flavin chromophore, which occurs at flavin position 4a, 5, and 8 (Hemmerich et al., 1973), three transient radical combination adducts ($R\text{-}HFl_{red}$) are postulated. As can be seen from the formulas, only the splitting of the 8 and 4a adduct yields Fl_{ox}, while from the 5 adduct HFl_{red}^- is derived. The latter is the end product of anaerobic long-term illumination.

Due to the small change of absorbance of $R\text{-}HFl_{red}$ and HFl_{red}^-, bond cleavage to yield HFl_{red}^- will escape detection. Formation of Fl_{ox} from 8-$R\text{-}HFl_{red}$ and 4a-$R\text{-}HFl_{red}$, however, should be observable experimentally, as demonstrated in Figure 5.

Considering now the fate of the NTA substrate, the NTA fragment RH, i.e., $>N\text{-}CH_3$, which results from pathways A and B in Scheme II, is found in long-term illumination up to a yield of 30% per photoreduced flavin (Armstrong et al., 1982). The NTA fragment R^+, i.e., $>N^+{=}CH_2$ of pathway C in Scheme II, readily solvolyzes according to

$$>N^+{=}CH_2 + H_2O \rightarrow >NH + CH_2O + H^+ \quad (12)$$

Scheme II: Mechanism of Flavin Photoreduction by NTA



and is found as formaldehyde in a yield larger than 90% per reduced flavin (Armstrong et al., 1982).

Comparison of the Photosubstrates. As confirmed experimentally, photoinduced charge separation should occur—with flavin being the electron acceptor—with substrates containing groups of low oxidation potential, like the tertiary amino group (Traber et al., 1981). From energetic reasons the photoinduced charge separation should be reversible in the dark, as detected in the Fl_{ox}/NO_2^- photosystem. So that an irreversible redox reaction can be obtained, competing processes have to be involved, like decarboxylation, as shown in the Fl_{ox}/EDTA and Fl_{ox}/NTA photoreaction, or deprotonation, as found in the Fl_{ox}/*N*-allylthiourea photoreaction (R. Traber, H. E. A. Kramer, and P. Hemmerich, unpublished results).

The fate of the irreversibly oxidized substrate radical is in our case now confined to a second oxidation step. Dismutation, as observed in electrochemical anodic oxidation experiments of aliphatic amines (Mann & Barnes, 1970), is disfavored for EDTA and NTA due to the lack of β-CH_2 groups in the radical fragments, which prevents enamine formation.

Instead, an internal 1,6-hydrogen shift follows in the EDTA radical fragment, yielding a more stable secondary radical. This is deduced from the product pattern in the overall reaction (Armstrong et al., 1982). 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 Fl_{ox}/NTA photoreaction is obtained (Armstrong et al., 1982).

While the NTA radical adds to the flavosemiquinone to yield a reduced flavin chromophore, the secondary EDTA radical fragment 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 HFl self-dismutation. Therefore, electron transfer to Fl_{ox}, which is then the only oxidant present at longer times, is the only decay mode for the EDTA radical fragment.

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Purification and Properties of a Presynaptically Acting Neurotoxin, Mandaratoxin, from Hornet (*Vespa mandarinia*)†

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ABSTRACT: A hornet (*Vespa mandarinia*) neurotoxin, mandaratoxin (MDTX), was purified by simple procedures with column chromatography made on Sephadex G-50 and CM-Sephadex by using an acetate buffer. The molecular weight of homogeneous MDTX was calculated to be approximately 20000 by gel filtration, $NaDodSO_4$ disc gel electrophoresis, and amino acid analysis. MDTX is a single-chain polypeptide. MDTX did not migrate electrophoretically in a basic buffer at pH 8.3 but did so when the buffer was acidic, at pH 4.3. The isoelectric point of the toxin was determined at 9.1 by isoelectric focusing. A relatively high amount of lysine was found in the amino acid analysis. $A^{1\%}_{280nm}$ was 15.1. Glucosamine and galactosamine were not detectable by amino acid analysis. MDTX had neither hemolytic nor enzymatic activity. The toxin was heat labile. By use of neuromuscular junctions of a lobster walking leg, it was found that the nanomole range of MDTX irreversibly blocked the excitatory postsynaptic potential without appreciable change in the resting conductance of the postsynaptic membrane. Intracellular recording from the presynaptic nerve fiber showed that MDTX blocked the action potential mainly by reducing the sodium current.

Much research has been carried out by using neurotoxins which block neuromuscular transmission in order to obtain information on the specific active sites and mechanisms involved in transmitter release (Ceccarelli & Clementi, 1979; Narahashi, 1974). Studies on the venom of *Hymenoptera* such as bees, wasps, and hornets have shown that the venom contains a variety of neurotoxic substances (Habermann, 1972; Piek, 1980; Kawai et al., 1980; Hori et al., 1977). However, due to the difficulty in collecting a sufficient amount of venom, research has not been carried out appreciably on the purification of neurotoxin or determination of active sites.

The giant hornet, *Vespa mandarinia*, which inhabits east and south Asia, is known as a very harmful insect since even one sting can result in death to man or domestic animals. A relatively large portion of the venom sac (ca. 20 mg fresh weight of the sac) of this species has facilitated the separation of the neuroactive components and elucidation of its action on a cellular level (Hori et al., 1977; Abe et al., 1979, 1980a).

The present study deals with a new polypeptide neurotoxin isolated from the venom of this hornet. We report the physical and chemical properties of this toxin and the specific mode of its action on the nerve membrane in blocking neuromuscular transmission. A preliminary account of this report has appeared elsewhere (Abe et al., 1980b).

Materials and Methods

Materials. The Sephadex G-50 (medium) and CM-Sephadex C-50 were purchased from Pharmacia Chemical Co. Sources of the standard proteins of sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide disc gel electrophoresis are as follows: bacitracin from Schwarz/Mann; ribonuclease from Boehringer; bovine serum albumin, ovalbumin, and chymotrypsinogen A from Miles Chemical Co. For isoelectric focusing, p*I* marker proteins containing cytochrome *c* from horse muscle and acetylated cytochrome *c* of several p*I* values, that is, 10.6, 9.7, 8.3, 6.4, 4.9, and 4.1, were purchased from Oriental Yeast Co. (Osaka). Iminodiacetic acid and ethylenediamine for the isoelectric focusing buffer were obtained from Tokyo Kasei Chemical Co. and Kishida Chemical Co., re-

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