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Photo-induced reduction of flavin mononucleotide in aqueous solutions

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Abstract

The photo-induced reduction of flavin mononucleotide (FMN) in aqueous solutions is studied by absorption spectra measurement under aerobic and anaerobic conditions. Samples without exogenous reducing agent and with the exogenous reducing agents ethylene-diamine-tetraacetic acid (EDTA) and dithiothreitol (DTT) are investigated. Under anaerobic conditions the photo-induced reduction with and without reducing agents is irreversible. Under aerobic conditions the photo-reduction without added reducing agent is small compared to the photo-degradation, and the photo-reduction of FMN by the reducing agents is reversible (re-oxidation in the dark). During photo-excitation of FMN the dissolved oxygen is consumed by singlet oxygen formation and subsequent chemical reaction. After light switch-off slow re-oxidation (slow absorption recovery) occurs due to air in-diffusion from surface. EDTA degradation by FMN excitation leads to oxygen scavenging. The quantum efficiencies of photo-reduction under aerobic and anaerobic conditions are determined. The re-oxidation of reduced FMN under aerobic conditions and due to air injection is investigated.

Keywords: Flavin mononucleotide (FMN); Ethylene-diamine-tetraacetic acid (EDTA); Dithiothreitol (DTT); Flavin photo-reduction; Aerobic photo-reduction; Anaerobic photo-reduction; Absorption spectroscopy; Flavin re-oxidation; Oxygen scavenging

1. Introduction

Flavins are blue-light absorbing dyes [1] with rich redox chemistry and photochemistry. Riboflavin (vitamin B_2), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) are cofactors in flavoproteins with biological activity in enzymes [2] and photoreceptors [3–5].

Under aerobic conditions the flavins are stable in the oxidized form [1,6–10]. They may be reduced chemically with dithionite [11,12], borohydride [13], hydrogen [14,15], or natural substrates [14,16]. The photo-reduction of flavins in the presence of electron donors is well established [17–19]. Photo-activation of flavins in air-saturated solutions with reducing agents converts them during light exposure to the semi and fully reduced form, but then they get re-oxidized in the dark. Under anaerobic conditions stable photo-reduction to the fully reduced form is achieved [14,16,20–29] even in the absence of exogenous reducing agents [22,24,29]. The involved mechanisms in the photo-reduction of flavins are discussed in [20–32]. It is generally accepted that the photo-excitation leads to triplet state population by intersystem crossing, and that the reduction occurs from the triplet state [21,22,24–32]. Some investigated reducing agents for photo-induced reduction of flavins are ethylene-diamine-tetraacetic acid (EDTA) [20,22–24,27–33], nitrilotriacetate [20], phenylacetic acid [26], indole-acetic acid [26,28], ascorbic acid (vitamin C) [28], and tryptophan [28].

In this paper we investigate the photo-reduction of FMN (flavin mononucleotide) in aqueous solutions at pH 8 under aerobic and anaerobic conditions. At pH 8 the oxidized form of FMN is in the neutral stage (FMN_{ox}), also the semi-reduced form is dominantly in the neutral stage

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(FMNH$^{\bullet}$), while the fully reduced form is in the anionic stage ($FMN_{red}H^{-}$) (see scheme 14 in [1], vol. 1). Studies have been carried out without reducing agent and with the reducing agents EDTA and dithiothreitol (DTT). The samples are excited with blue light and absorption spectra are recorded after certain times of exposure. The quantum efficiency of photo-reduction is determined, and the re-oxidation of the samples is investigated.

2. Experimental

The flavin FMN (riboflavin 5′-monophosphate sodium salt dehydrate, purity ≈ 95% tested by HPLC, Sigma biochemicals #F8399) and the reducing agents EDTA (ethylene-diamine-tetraacetic acid disodium salt dehydrate, purity 99.995%, Aldrich #431788) and DTT (DL-dithiothreitol, purity >99% tested by titration, Aldrich #D0632) were purchased from Sigma–Aldrich and were used without further purification. Their structural formulae are shown in Fig. 1 together with structural formulae of $FMN_{red}H_2$ and $FMN_{red}H^{-}$. FMN is dissolved in aqueous sodium phosphate buffer pH 8 containing 10 mM NaCl. The reducing agents were added to prepared FMN solution without pH readjustment. The oxygen content in aerobic aqueous solutions is 0.29 mM at 20 °C and 0.27 mM at 25 °C [34]. Anaerobic samples were prepared by bubbling with argon (impurity content $< 10^{-5}$) for at least 30 min in a nitrogen filled glove-box. The solubility of EDTA in water is limited to about 1.7 mM. DTT is good soluble in water.

The photo-reduction setup is depicted in Fig. 2. The aerobic samples were excited in a small-volume cell

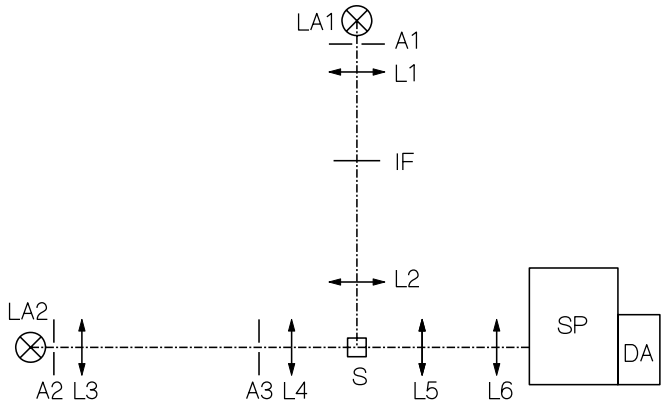
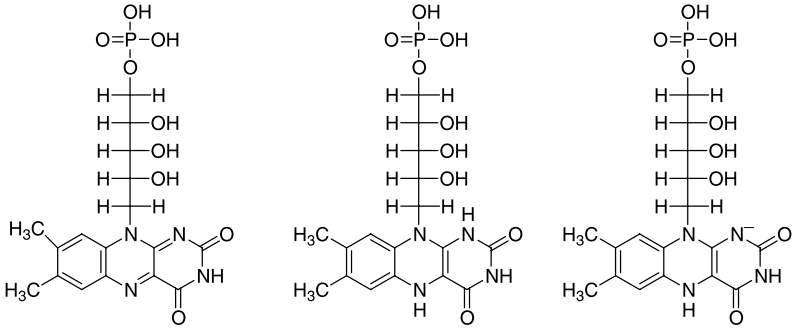


Fig. 2. Photo-reduction experimental setup: LA1, mercury lamp (250 W) or xenon lamp (450 W) for excitation; LA2, tungsten lamp or deuterium lamp for probing; A1–A3, apertures; L1–L6, lenses; IF, interference filter; S, sample; SP, spectrometer; DA, diode-array detection system.

(1.5 mm × 1.5 mm × 5 mm) with a 250 W high-pressure mercury lamp through an appropriate interference filter centred at 406 nm (spectral half-width 10 nm, Hg-lamp could be focused to the small volume cell aperture to obtain sufficient excitation intensity). The anaerobic samples were excited with a 450 W high-pressure xenon lamp in a cell of 1 mm thickness, 1 cm width, and 1 cm filled height through a broad-band interference filter (350–440 nm). The larger cell was used to facilitate the de-aeration by argon bubbling; and the xenon lamp with the broadband interference filter was needed to supply sufficient homogenous excitation intensity for the larger cell area. The sample absorption was probed by wide-wave-



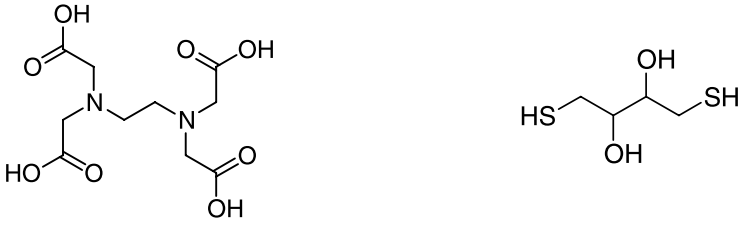


Fig. 1. Structural formulae of FMN_{ox}, $FMN_{red}H_2$, $FMN_{red}H^{-}$, EDTA, and DTT. (FMN) Sum formula: $C_{17}H_{21}N_4O_9P$; molar mass: $M_m = 456.3\ g\ mol^{-1}$. (EDTA) Formula: $(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$; molar mass: $M_m = 292.24\ g\ mol^{-1}$; melting point: $\vartheta_m = 250\ °C$. (DTT) Formula: $HSCH_2CH(OH)CH(OH)CH_2SH$; molar mass: $M_m = 154.25\ g\ mol^{-1}$; melting point: $\vartheta_m = 41–44\ °C$.

length-range transmission measurement with a tungsten lamp or a deuterium lamp and a spectrometer – silicon diode-array detection system. The samples were long-time exposed, and transmission spectra were recorded at certain time intervals.

The quantum efficiency of photo-reduction, ϕ_{red}, is extracted from time dependent absorption changes due to light exposure at a wavelength of strong FMN_{ox} absorption and small reduced FMN (both anionic, $FMN_{red}H^-$, and neutral, $FMN_{red}H_2$) absorption ($\lambda_{pr} = 470$ nm). ϕ_{red} is given by

$$\phi_{red} = \frac{\Delta N_{red}}{\Delta n_{ph,abs}}, \tag{1}$$

where $\Delta N_{red} = \int_0^\ell N_{red}(z)\,dz$ is the length-integrated number density of molecules transferred to the reduced state, and $\Delta n_{ph,abs}$ is the number density of absorbed photons. ΔN_{red} is given by

$$\Delta N_{red} = \ell \frac{\bar{\alpha}_{pr}(t) - \bar{\alpha}_{pr}(t+\Delta t)}{\sigma_{a,pr} - \sigma_{a,red,pr}} \tag{2}$$

where ℓ is the sample length, $\bar{\alpha}_{pr}(t) = -\ln(T_{pr}(t))/\ell$ is the length-averaged absorption coefficient of the sample at probe wavelength λ_{pr} at time t, Δt is the considered time interval, $\sigma_{a,pr}$ is the absorption cross-section of FMN_{ox} at λ_{pr}, and $\sigma_{a,red,pr}$ is the absorption cross-section of the fully reduced FMN ($FMN_{red}H^-$ for pH > 6.7, or $FMN_{red}H_2$ for pH 0–6.7 [1]) at λ_{pr}. $\Delta n_{ph,abs}$ is given by

$$\begin{aligned}\Delta n_{ph,abs} &= \frac{I_{exc}\Delta t}{h\nu_{exc}}(1 - \overline{T}_{exc}) \\ &= \frac{I_{exc}\Delta t}{h\nu_{exc}}\{1 - \exp[-\bar{\alpha}(\lambda_{exc})\ell]\},\end{aligned} \tag{3}$$

where I_{exc} is the incident excitation intensity at the excitation frequency $\nu_{exc} = c_0/\lambda_{exc}$ (c_0 is the vacuum light velocity), $\overline{T}_{exc} = [T(\lambda_{exc}, t) + T(\lambda_{exc}, t+\Delta t)]/2$ is the average transmission at the excitation wavelength in the time interval Δt, $\bar{\alpha}$ is the corresponding average absorption coefficient in the time interval Δt.

3. Results

The absorption cross-section spectrum of FMN in pH 8 aqueous solution in the dark is shown in Fig. 3a (dashed curve). Within the experimental accuracy the spectrum is unchanged by adding of the reducing agents EDTA or DTT in the transparency region of these substrates. The absorption cross-section spectra of fully reduced neutral FMN ($FMN_{red}H_2$, dotted curve) and of fully reduced anionic FMN ($FMN_{red}H^-$, dash-dotted curve) are included (curves taken from [1]). The effective absorption cross-section spectrum of fully reduced FMN obtained by long-time light exposure of FMN in 1 mM EDTA and 10 mM DTT under anaerobic conditions is included (taken from Fig. 10a below, absolute scale obtained from isobestic point at $\lambda = 335$ nm). The spectrum resembles that of

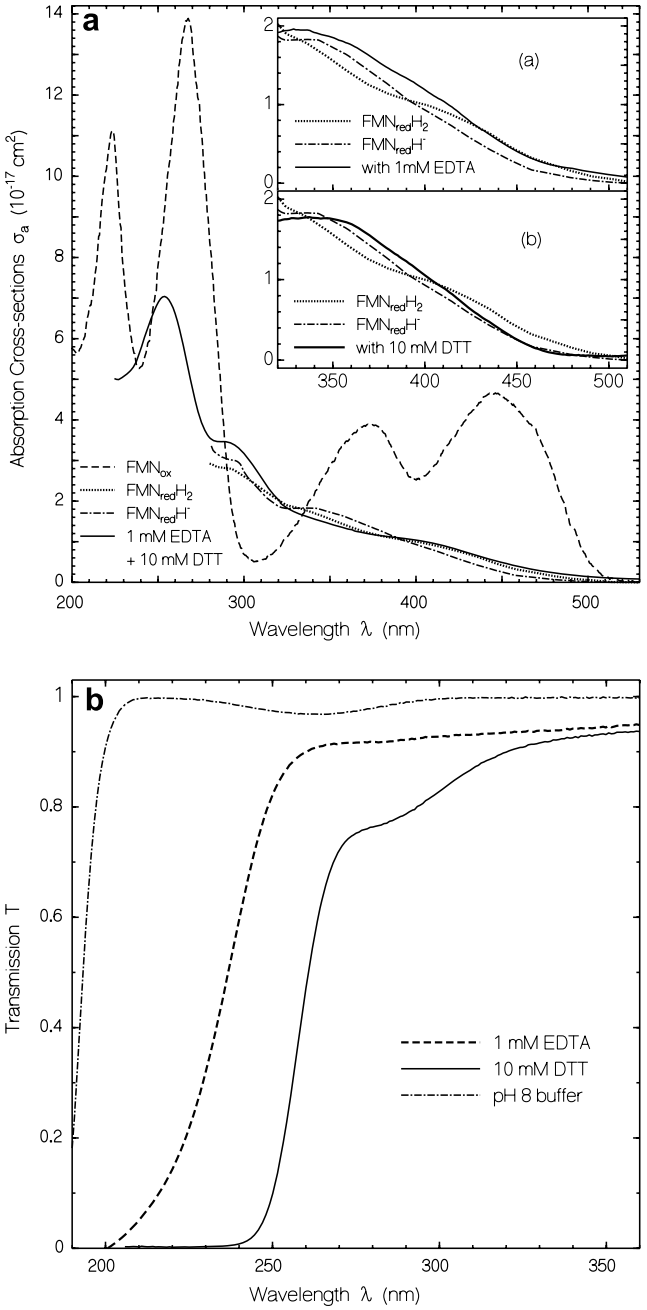


Fig. 3. (a) Absorption cross-section spectra of FMN (oxidized state, FMN_{ox}) in aqueous phosphate buffer solution at pH 8 (dashed curve), and of photo-reduced FMN in aqueous pH 8 phosphate buffer with additional 1 mM EDTA and 10 mM DTT (solid curve, taken from Fig. 10a, $t_{exp} = 20$ min, $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2}). Absorption cross-section spectra of fully reduced neutral FMN ($FMN_{red}H_2$, dotted curve) and of fully reduced anionic FMN ($FMN_{red}H^-$, dash-dotted curve) are included (curves taken from [1], vol. 1). Insert (a) effective absorption cross-section spectrum of long-time-exposed FMN in aqueous solution with 1 mM EDTA (solid curve, taken from Fig. 6a, $t_{exp} =$ 20 min, $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2}). Insert (b) effective absorption cross-section spectrum of long-time-exposed FMN in aqueous solution with 10 mM DTT (solid curve, taken from Fig. 8a, $t_{exp} =$ 25 min, $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2}). (b) Transmission spectra of phosphate aqueous buffer at pH 8 (mixture of 10 mM Na_2HPO_4 and 10 mM NaH_2PO_4, additional 10 mM NaCl) versus bi-distilled water, of 1 mM EDTA in pH 8 buffer versus pH 8 buffer, and of 10 mM DTT in pH 8 buffer versus pH 8 buffer. Sample thickness is 1 cm in all cases.

$FMN_{red}H_2$ (pH of solution lowered below pH 6.7 due to addition of 1 mM EDTA). The deviation below 320 nm is thought to be caused by DTT absorption contribution. In the insert (a) the effective absorption cross-section spectrum of long-time exposed anaerobic FMN in 1 mM EDTA is shown (taken from Fig. 6a, absolute scale obtained from isobestic point at $\lambda = 337.4$ nm). In the long-wavelength range ($\lambda > 420$ nm) it resembles the absorption cross-section spectrum of $FMN_{red}H_2$. At shorter wavelength the transmission is increased because of absorption contribution of photo-degraded FMN (mainly lumichrome [35]). In the insert (b) the effective absorption cross-section of long-time exposed anaerobic FMN in 10 mM DTT is depicted (taken from Fig. 8a, absolute scale obtained from isobestic point at $\lambda = 335.6$ nm). In the long-wavelength range ($\lambda > 420$ nm) it resembles the absorption cross-section spectrum of $FMN_{red}H^-$. At shorter wavelength the transmission again is increased because of absorption contribution of photo-degraded FMN (mainly lumichrome [35]).

The transmission spectra of 1 cm thick samples of pH 8 buffer solution, 1 mM EDTA, and 10 mM DTT in pH 8 buffer solution are shown in Fig. 3b.

3.1. Photo-reduction without exogenous reducing agent

The photo-reduction of FMN in aqueous pH 8 buffer without exogenous reducing agent under anaerobic conditions is shown in Figs. 4a, b, and 5a. The FMN sample was bubbled with argon for 30 min before transmission measurement. In Fig. 4a the sample was excited at $\lambda_{exc} = 350$–440 nm with an input intensity of $I_{exc} = 8.4 \times 10^{-3}$ W cm^{-2}. The FMN_{ox} absorption bands centred at 450 and 375 nm decreased during exposure. The shape of the absorption spectra changed due to the formation of fully reduced anionic FMN, $FMN_{red}H^-$. No formation of FMN-semiquinone, FMNH$^{\bullet}$, is observed (no absorption build-up in the 500–600 nm region) under our experimental conditions.

After light switch-off, in the dark the absorption development is shown in Fig. 4b. Within a time period of 20 min practically no absorption recovery from $FMN_{red}H^-$ to FMN_{ox} was observed. Then 3 cm^3 of air were bubbled through the sample in a time period of 30 s. Within this time the absorption recovered. $FMN_{red}H^-$ re-oxidized back to FMN_{ox}. After that the absorption remained constant. The time constant of re-oxidation is less than 30 s. No complete absorption recovery occurred since some photo-degradation took place during the light exposure [35]. The changes of the absorption shape indicate the formation of some lumichrome [35].

The temporal development of the absorption at $\lambda_{pr} = 470$ nm during light exposure with $I_{exc} = 8.4 \times 10^{-3}$ W cm^{-2} at $\lambda_{exc} = 350$–440 nm, after light switch-off, and after air injection is displayed in Fig. 5a.

Analysis of the initial absorption decrease due to light exposure (Fig. 5a) gives an initial quantum yield of photo-reduction of FMN_{ox} to $FMN_{red}H^-$ of $\phi_{red,0} \approx 0.022 \pm 0.01$ (application of Eqs. (1)–(3)).

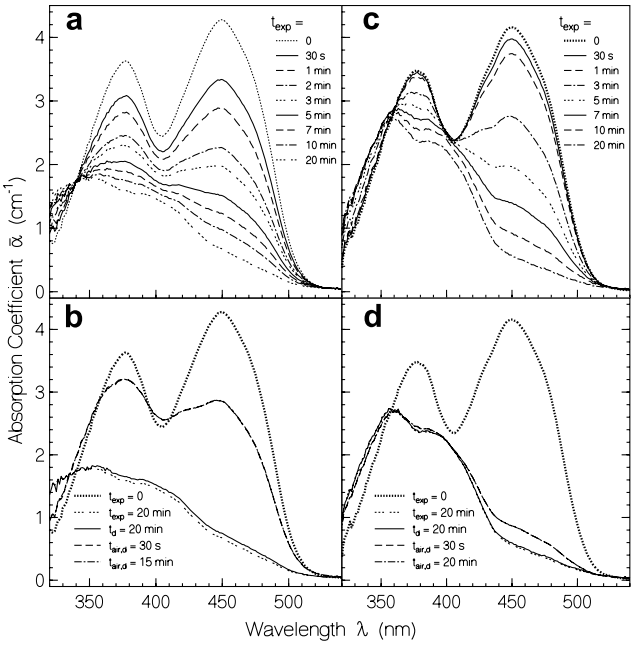


Fig. 4. Photo-reduction of FMN in pH 8 buffer without added reducing agent. Concentration $C = 1.53 \times 10^{-4}$ mol dm^{-3}. Excitation wavelength, $\lambda_{exc} = 350$–440 nm, excitation intensity, $I_{exc} = 8.4 \times 10^{-3}$ W cm^{-2}. Exposure times, t_{exp}, dark times after light switch-off, t_d, and dark times after air injection, $t_{air,d}$, are given in the figure. Absorption coefficient spectra, $\tilde{\alpha}(\lambda) = -\ln[T(\lambda)]/\ell$, are shown ($T$ is transmission, $\ell = 1$ mm is sample length). (a) Photo-excitation under anaerobic conditions. (b) Absorption recovery in the dark after light switch-off and after air injection (3 cm^3 within 30 s) under anaerobic conditions. (c) Photo-excitation under aerobic conditions. (d) Absorption recovery in the dark after light switch-off and after air injection (3 cm^3 within 30 s) under aerobic conditions.

The photo-reduction of FMN in aqueous pH 8 buffer without exogenous reducing agent under air-saturated conditions is shown in Figs. 4c, d, and 5b. The sample is excited at $\lambda_{exc} = 350$–440 nm with an input intensity of $I_{exc} = 8.4 \times 10^{-3}$ W cm^{-2}. The FMN_{ox} absorption band centred at 450 nm decreases with exposure time. The absorption at 380 nm decreases only slightly. The formed absorption band in the 350–400 nm region belongs to the photoproduct lumichrome [35].

After light switch-off, in the dark the absorption spectrum remains unchanged (observed over a period of 20 min). Air injection (3 cm^3 in 30 s) results in small prompt absorption rise in the 450 nm region (FMN_{ox} absorption band). This absorption rise indicates that the photo-excitation converted about 8% of FMN_{ox} to $FMN_{red}H^-$, which is re-oxidated in the presence of oxygen. At the end of exposure, after light switch-off, only a negligible rise of absorption at $\lambda_{pr} = 470$ nm is observed. It is thought that during photo-excitation the originally dissolved oxygen is consumed by chemical reaction: photo-excitation of FMN causes triplet-state formation [36–39], ^{3}FMN causes singlet oxygen formation [38,40], $^1O_2$ is very reactive and is consumed by chemical reaction.

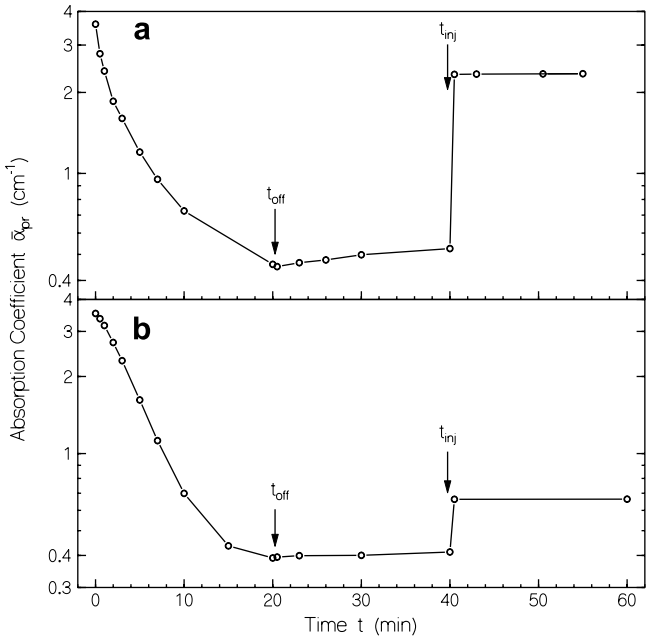


Fig. 5. Temporal development of absorption coefficient, $\bar{\alpha}_{pr}$, at probe wavelength $\lambda_{pr} = 470$ nm for photo-reduction of FMN in pH 8 buffer without exogenous reducing agent. Experimental situation of Fig. 4 is valid. Time positions of light switch-off, t_{off}, and of air-injection, t_{inj}, are indicated. (a) Anaerobic conditions. (b) Aerobic conditions.

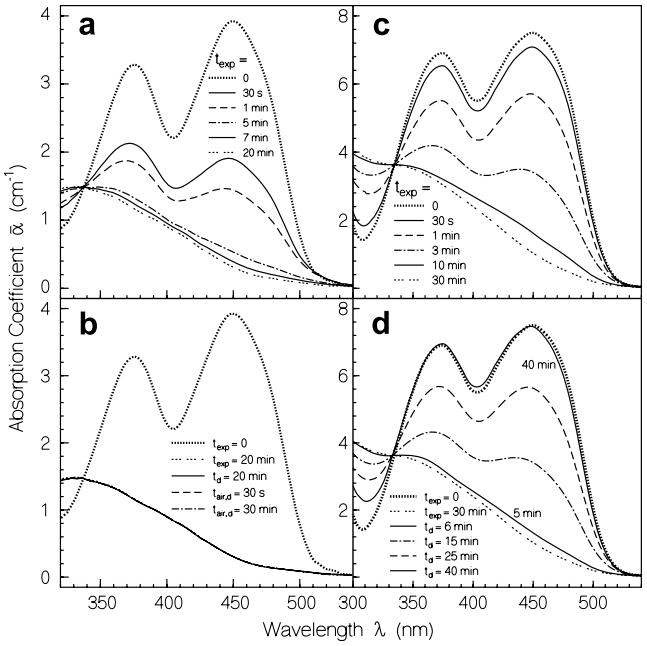


Fig. 6. Photo-reduction of FMN in pH 8 buffer with 1 mM EDTA. (a) Photo-excitation under anaerobic conditions. $C = 1.4 \times 10^{-4}$ mol dm^{-3}. $\lambda_{exc} = 350$–440 nm. $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2}. Sample length $\ell = 1$ mm. (b) Absorption recovery in the dark after light switch-off and after air injection (3 cm^3 within 30 s) under anaerobic conditions. (c) Photo-excitation under aerobic conditions. $C = 2.68 \times 10^{-4}$ mol dm^{-3}. $\lambda_{exc} = 407$ nm. $I_{exc} = 3.5 \times 10^{-3}$ W cm^{-2}. $\ell = 1.5$ mm. (d) Absorption recovery in the dark after light switch-off under aerobic conditions.

3.2. Photo-reduction with EDTA

The light dependent absorption behaviour of FMN in pH 8 buffer solution with added EDTA to 1 mM concentration under anaerobic conditions is shown Figs. 6a, b, and 7a. Excitation of the sample at $\lambda_{exc} = 350$–440 nm with $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2} causes already strong reduction to $FMN_{red}H_2$ within 30 s of exposure (Fig. 6a). After light switch-off no recovery of $FMN_{red}H_2$ to FMN_{ox} is observed (no absorption change within 20 min of observation in the dark, Fig. 6b). Air injection (3 cm^3 in 30 s) after 20 min dark time does not change the absorption (no re-oxidation). Obviously the oxygen entered by the bubbling procedure is scavenged by the EDTA photo-products formed during photo-excitation of FMN.

The temporal development of the absorption at $\lambda_{pr} = 470$ nm due to light-exposure, keeping in the dark, and injecting of air is shown in Fig. 7a. Application of Eqs. (1)–(3) to the initial light-induced absorption reduction gives a quantum efficiency of photo-reduction of $\phi_{red,0} \approx 0.15 \pm 0.04$.

The light dependent absorption behaviour of FMN in pH 8 buffer solution with added EDTA to 1 mM concentration under aerobic conditions is shown Figs. 6c, d, and 7b. The sample is excited at $\lambda_{exc} = 407$ nm with $I_{exc} = 3.5 \times 10^{-3}$ W cm^{-2}. Within the first 30 s the photo-reduction is small and then it becomes strong (Fig. 6c). Probably initially the re-oxidation is strong until the oxygen is consumed. After light switch-off the absorption recovery (re-oxidation) starts slowly and then speeds up

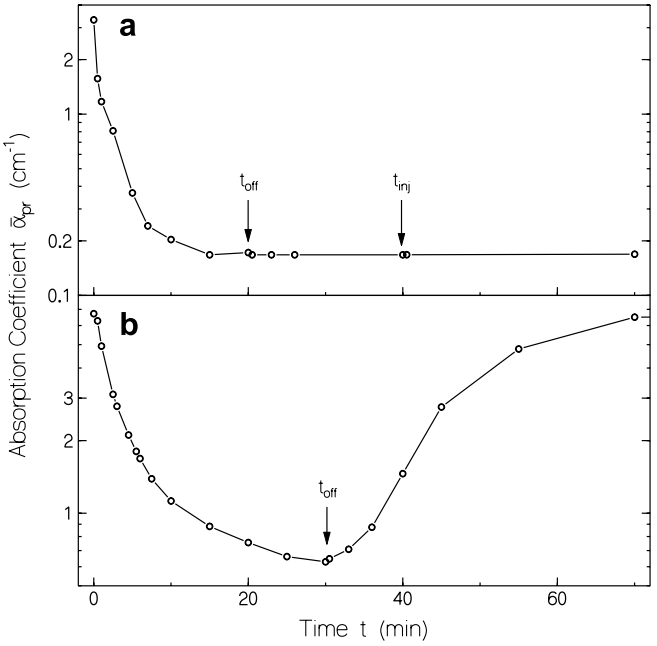


Fig. 7. Temporal development of absorption coefficient, $\bar{\alpha}_{pr}$, at $\lambda_{pr} = 470$ nm for photo-reduction of FMN in pH 8 buffer with 1 mM EDTA. Experimental situation of Fig. 6 is valid. (a) Anaerobic conditions. (b) Aerobic conditions.

(Fig. 6d). The initial absorption of FMN_{ox} is nearly fully reached within 40 min (only small photo-degradation). The re-oxidation is thought to be caused by in-diffusing

oxygen. The slow start of re-oxidation is thought to be due to the presence of oxygen scavenging photoproducts of EDTA. The temporal development of the absorption at the probe wavelength $\lambda_{pr} = 470$ nm is shown in Fig. 7b.

The initial quantum yield of photo-reduction is calculated (Eqs. (1)–(3)) to be $\phi_{red,0} \approx 0.016$. In the time range from 30 s to 1 min of exposure the efficiency of photo-reduction increased to $\phi_{red} \approx 0.072$. The 1/e-time of recovery (fraction of $1 - \exp(-1) = 0.632$ of the reduced molecules returned to the oxidized state) is $t_{rec,1/e} \approx 23.5$ min.

3.3. Photo-reduction with DTT

The light dependent absorption behaviour of FMN in pH 8 buffer solution containing 10 mM DTT under anaerobic conditions is shown in Figs. 8a–c, and 9a. Excitation of the sample at $\lambda_{exc} = 350$–440 nm with $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2} causes efficient reduction to $FMN_{red}H^-$ (Fig. 8a). After light switch-off no recovery of $FMN_{red}H^-$ to FMN_{ox} was observed within a time period of 20 min (Fig. 8b). Air bubbling through the sample (3 cm^3 during 30 s) causes complete re-oxidation of $FMN_{red}H^-$ to FMN_{ox} (complete absorption recovery) during the bubbling period (Fig. 8c). The temporal development of the absorption at $\lambda_{pr} = 470$ nm during light exposure, after light switch-off, and due to air-injection is shown in Fig. 9a.

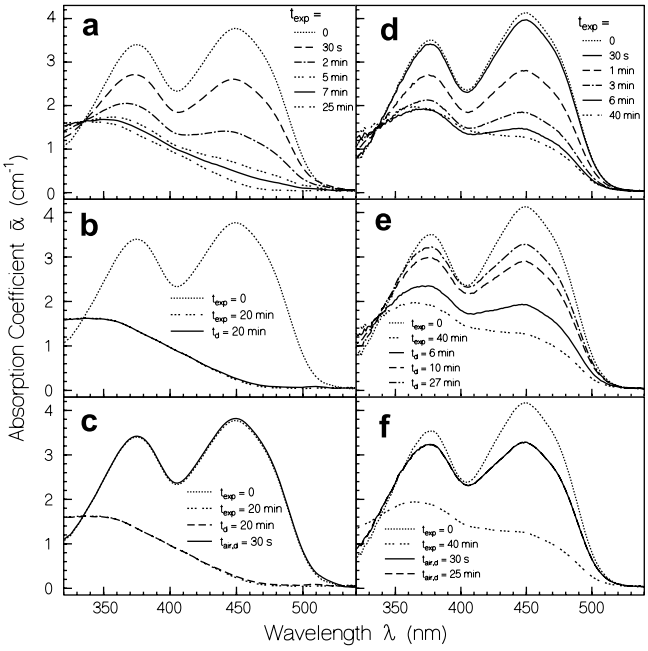


Fig. 8. Photo-reduction of FMN in pH 8 buffer with 10 mM DTT. $\lambda_{exc} = 350$–440 nm. $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2}. Sample length $\ell = 1$ mm. (a) Photo-excitation under anaerobic conditions. $C = 1.35 \times 10^{-4}$ mol dm^{-3}. (b) Absorption recovery in the dark after light switch-off under anaerobic conditions. (c) Absorption spectra measured before light exposure, after 20 min of light exposure, then after 20 min in the dark, and finally 30 s after air injection (3 cm^3 within 30 s) under anaerobic conditions. (d) Photo-excitation under aerobic conditions. $C = 1.48 \times 10^{-4}$ mol dm^{-3}. (e) Absorption recovery in the dark after light switch-off under aerobic conditions. (f) Absorption spectra measured before light exposure, after 40 min of light exposure, at the end of air injection (3 cm^3 within 30 s) immediately after light switch-off, and 25 min later under anaerobic conditions (both curves not distinguishable).

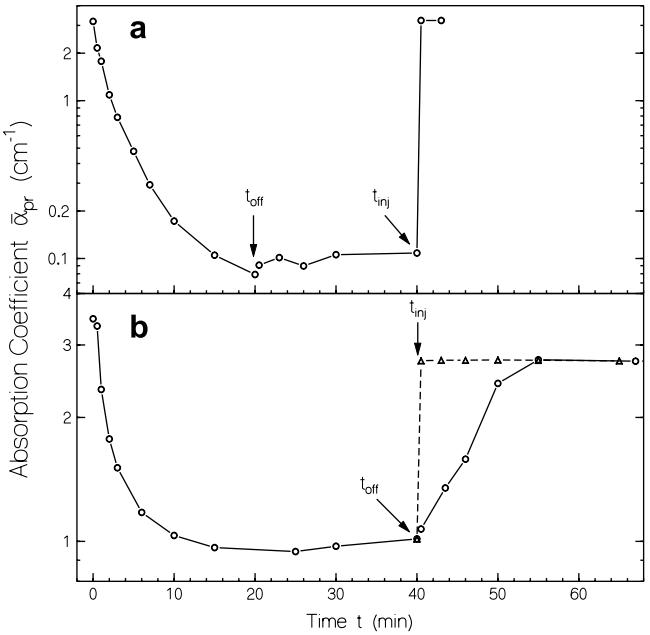


Fig. 9. Temporal development of absorption coefficient, $\bar{\alpha}_{pr}$, at $\lambda_{pr} = 470$ nm for photo-reduction of FMN in pH 8 buffer with 10 mM DTT. Experimental situation of Fig. 8 is valid. (a) Anaerobic conditions. (b) Aerobic conditions. Solid line connected circles: No air injection after light switch-off. Dashed line connected triangles: Air injection at moment of light switch-off.

An initial quantum efficiency of photo-reduction of $\phi_{red,0} = 0.08 \pm 0.02$ is estimated by use of Eqs. (1)–(3). The addition of 10 mM DTT increased the initial quantum efficiency of photo-reduction from $\phi_{red,0} \approx 0.022$ solution without DTT to the value of $\phi_{red,0} \approx 0.08$ (nearly fourfold increase in efficiency).

The light dependent absorption behaviour of FMN in pH 8 buffer solution containing 10 mM DTT under aerobic conditions is shown in Figs. 8d–f, and 9b. The sample is excited at $\lambda_{exc} = 350$–440 nm with $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2}. Within the first 30 s the photo-reduction is small and then it becomes strong (Fig. 8d). Again the slow start of FMN_{ox} absorption decrease is probably due to efficient re-oxidation until the dissolved oxygen is consumed. After light switch-off the absorption recovery (re-oxidation) starts (Fig. 8e). The initial absorption strength of FMN_{ox} is not completely reached because of some accompanying photo-degradation [35]. The re-oxidation is thought to be caused by in-diffusing oxygen. In Fig. 8f the photo-excitation is repeated as in Fig. 8d (40 min of light exposure, data not collected during exposure), and immediately after light switch-off air is injected for 30 s (injected volume 3 cm^3). Within this 30 s complete $FMN_{red}H^-$ re-oxidation to FMN_{ox} takes place (no further change in absorption was observed over a period of 25 min).

The temporal development of the absorption at the probe wavelength $\lambda_{pr} = 470$ nm due to photo-excitation and light switch-off without air bubbling (circles) and due to photo-excitation with air injection at the moment of

light switch-off (triangles, data not collected during exposure) is shown in Fig. 9b.

The initial quantum yield of photo-reduction within the first 30 s of exposure is found to be $\phi_{red,0} \approx 0.0085$ (Eqs. (1)–(3)). In the time range from 30 s to 1 min of exposure the efficiency of photo-reduction increased to $\phi_{red} \approx 0.071$. The 1/e- time of recovery after light switch-off without air-bubbling is $t_{rec,1/e} \approx 8.5$ min (in-diffusion of oxygen from air–liquid interface). The re-oxidation time due to air injection could not be resolved under our experimental conditions ($t_{reox} < 10$ s).

3.4. Photo-reduction with EDTA and DTT

The light dependent absorption behaviour of FMN in pH 8 buffer solution with added 1 mM EDTA and 10 mM DTT under anaerobic conditions is shown in Figs. 10a, b, and 11a. Excitation of the sample at $\lambda_{exc} = 350$–440 nm with $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2} causes efficient reduction to $FMN_{red}H_2$ (Fig. 10a). After light switch-off only a very small absorption recovery is observed (very small re-oxidation of $FMN_{red}H_2$ to FMN_{ox}) within a time period of 20 min (Fig. 10b). Air bubbling through the sample (3 cm^3 during 30 s) causes only a very tiny absorption rise (Fig. 10b) showing the oxygen scavenging effect of degraded EDTA. The temporal development of the absorption at $\lambda_{pr} = 470$ nm during light exposure, after light switch-off, and due to air-injection is shown in Fig. 11a.

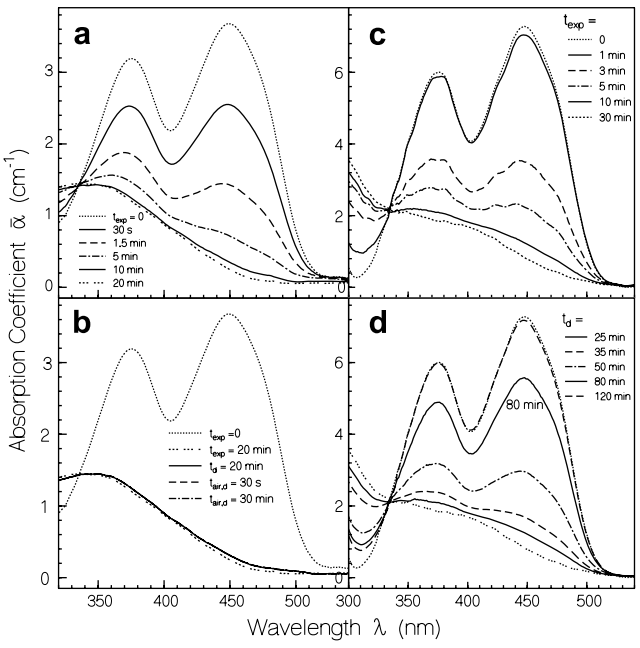


Fig. 10. Photo-reduction of FMN in pH 8 buffer with 1 mM EDTA and 10 mM DTT. (a) Photo-excitation under anaerobic conditions. $C = 1.31 \times 10^{-4}$ mol dm^{-3}. $\lambda_{exc} = 350$–440 nm. $I_{exc} = 3.1 \times 10^{-3}$ W cm^{-2}. Sample length $\ell = 1$ mm. (b) Absorption recovery in the dark after light switch-off and after air injection (3 cm^3 within 30 s) under anaerobic conditions. (c) Photo-excitation under aerobic conditions. $C = 2.6 \times 10^{-4}$ mol dm^{-3}. $\lambda_{exc} = 407$ nm. $I_{exc} = 3.5 \times 10^{-3}$ W cm^{-2}. $\ell = 1.5$ mm. (d) Absorption recovery in the dark after light switch-off under aerobic conditions (dotted line: $t_{exp} = 0$, triple-dotted line: $t_{exp} = 30$ min).

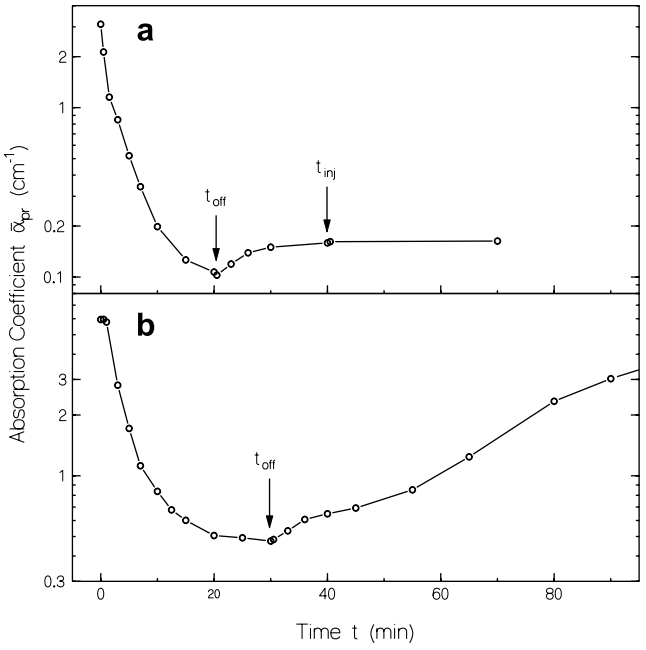


Fig. 11. Temporal development of absorption coefficient, $\bar{\alpha}_{pr}$, at $\lambda_{pr} = 470$ nm for photo-reduction of FMN in pH 8 buffer with 1 mM EDTA and 10 mM DTT. Experimental situation of Fig. 10 is valid. (a) Anaerobic conditions. (b) Aerobic conditions.

An initial quantum efficiency of photo-reduction of $\phi_{red,0} \approx 0.08 \pm 0.01$ is estimated. The photo-reduction efficiency is a factor of two smaller than the photo-reduction efficiency of 1 mM EDTA alone and is similar to the photo-reduction efficiency of 10 mM DTT alone.

The light dependent absorption behaviour of FMN in pH 8 buffer solution containing 1 mM EDTA and 10 mM DTT under aerobic conditions is shown in Figs. 10c, d, and 11b. The sample is excited at $\lambda_{exc} = 407$ nm with $I_{exc} = 3.5 \times 10^{-3}$ W cm^{-2}. Efficient photo-reduction starts after about 1 min of light exposure. Within this time it is thought that photo-induced oxygen consumption by EDTA and DTT takes place via triplet excited FMN and singlet oxygen generation. The delay in photo-reduction is longer in the presence of EDTA and DTT together than in the cases where only either EDTA or DTT is present indicating some photochemical effect between EDTA and DTT. After light switch-off the absorption recovers slowly to the initial absorption strength (complete re-oxidation). The slow absorption recovery is thought to be due to the oxygen scavenging effect of generated photoproducts by EDTA reaction with triplet FMN. The combined presence of EDTA and DTT slows down the recovery much stronger than either EDTA or DTT alone. The temporal development of the absorption at $\lambda_{pr} = 470$ nm during light exposure and after light switch-off is shown in Fig. 11b.

The initial quantum yield of photo-reduction within the first minute of exposure is $\phi_{red,0} \approx 0$. In the time range from 1 to 3 min of light exposure the quantum yield of

photo-reduction is determined (Eqs. (1)–(3)) to be $\phi_{red} \approx 0.038$. This efficiency is approximately a factor of two less then in the presence of 1 mM EDTA or 10 mM DTT alone. The 1/e-time of recovery after light switch-off is $t_{rec,1/e} \approx 72$ min.

4. Discussion

The quantum yields of photo-reduction and the 1/e-times of re-oxidation under anaerobic and aerobic conditions are collected in Table 1. The dynamics of photo-reduction under anaerobic and aerobic conditions is discussed below.

4.1. Anaerobic photo-reduction

Even without exogenous reducing agent, blue-light photo-reduction of FMN_{ox} to $FMN_{red}H^-$ was observed in aqueous solution at pH 8. The photo-reduction follows formally the over-all reaction

$$FMN_{ox} + 2H^{\cdot} + OH^- + h\nu \rightarrow FMN_{red}H^- + H_2O \qquad (R1)$$

A moderate quantum yield of photo-reduction of $\phi_{red,0} \approx 0.022$ was obtained. Blue-light absorption excites the flavin in the oxidized state, Fl_{ox}, from the singlet ground-state to a singlet excited state according to $Fl_{ox} + h\nu \rightarrow Fl^*_{ox}$. Part of the singlet excited molecules relax to the triplet state by singlet–triplet intersystem crossing (quantum yield of triplet formation ϕ_{isc}) in the process $Fl^*_{ox} \rightarrow {}^3Fl_{ox}$. The triplet-state lifetime of FMN is about $\tau_T \approx 11$ μs [41]. The reduction of Fl_{ox} is thought to occur from the triplet state. It is likely that the full flavin reduction proceeds via flavin–semiquinone formation ($Fl_{ox} + H^{\cdot} \rightarrow FlH^{\cdot}$) and fast flavin–semiquinone disproportionation ($2\,FlH^{\cdot} \rightarrow Fl_{ox} + Fl_{red}H_2$). At pH 8 the neutral hydroquinone form gets anionic ($FMN_{red}H_2 + OH^- \rightarrow FMN_{red}H^- + H_2O$). On our time scale with our excitation intensities (first data point measured after 30 s of exposure) no FMN semiquinone formation was observed. The photo-reduction of de-aerated riboflavin was studied in [28] by laser flash-photolysis. There the photo-reduction was shown to proceed through triplet–triplet annihilation via short-lived neutral semiquinone radical formation ($FlH^{\cdot}$).

The photo-reduction of de-aerated riboflavin was studied in [21,22,25,28,42–44]. It was proposed that water is the donor of hydrogen and that H_2O_2 is generated by photo-excitation [42]. In [21,22,43,44] evidence is given that the ribityl rest of riboflavin itself is the hydrogen donor. For lumiflavin (has no ribityl chain) no photo-reduction was observed [44]. In studies on the photo-degradation of FMN in aqueous solution under aerobic conditions it was found that FMN oxidized to formyllumiflavin and lumiflavin-hydroxy-acetaldehyde and formed dihydroxymethyllumiflavin as well as lumichrome [35].

The photo-reduction of FMN_{ox} in pH 8 buffer with 1 mM EDTA occurs according to a formal overall reaction [30]

$$FMN_{ox} + 2EDTA + h\nu \rightarrow FMN_{red}H_2 + \text{Photoproducts}. \qquad (R2)$$

EDTA delivers the two needed hydrogen atoms. It lowers the pH to below $pK_a = 6.7$ for dominant fully reduced neutral FMN formation. The details of the photo-reaction of FMN_{ox} with EDTA are rather complex, they are described in [27,30–32]. EDTA is irreversibly modified (indicated by Photoproducts) with CO_2 release and hydrolysis to glyoxylate. In [29] it was shown by time-resolved resonance Raman and absorption studies that an electron is abstracted from EDTA by FMN_{ox} in the triplet state to generate the anionic FMN–semiquinone ($^3FMN_{ox} + e^- \rightarrow FMN^{\cdot -}$) which is converted into $FMNH^{\cdot}$ by protonation when the pH is smaller than 8.3. $FMNH^{\cdot}$ quickly disproportionate into FMN_{ox} and $FMN_{red}H_2$ below pH 6.7.

Limited air injection into the photo-reduced FMN sample with EDTA led to no re-oxidation of $FMN_{red}H_2$ to FMN_{ox} showing the oxygen scavenging effect of the formed EDTA photoproducts. In the case of FMN photo-excitation EDTA acts as an oxygen scavenger (chemical oxygen getter [27]).

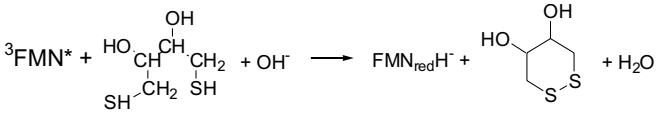
In the presence of 1 mM EDTA a quantum efficiency of FMN photo-reduction of $\phi_{red,0} = 0.15 \pm 0.04$ was measured. This means that nearly each second molecule photo-excited to the triplet state is reduced to $FMN_{red}H_2$ by EDTA. The quantum yield of triplet formation of FMN in neutral aqueous solution is in the range of 0.38 (measured for riboflavin [36]) to 0.6 [37–39].

The photo-reduction of FMN_{ox} in pH 8 buffer with 10 mM DTT under anaerobic conditions was found to be quite effective with an initial quantum yield of photo-reduction of $\phi_{red,0} = 0.08 \pm 0.02$. DTT (dithiothreitol) eas-

Table 1
Photo-reduction of FMN at room temperature in phosphate buffer pH 8, 10 mM NaCl under anaerobic and aerobic conditions, with and without added reducing agents

Parameter	No exogenous reducing agent	1 mM EDTA	10 mM DTT	1 mM EDTA and 10 mM DTT
Anaerobic				
$\phi_{red,0}$	0.022 ± 0.01	0.15 ± 0.04	0.08 ± 0.02	0.08 ± 0.02
Aerobic				
$\phi_{red,0}$		0.016 ± 0.003	0.0085 ± 0.002	≈0
$\phi_{red,max}$		0.072 ± 0.01	0.071 ± 0.02	0.038 ± 0.005
$t_{rec,1/e}$ (min)		23.5	8.5	72

ily oxidizes by cyclisation to [1,2]-dithiane-4,5-diol (DTTc) [45]. The photo-reduction of FMN may be understood by first singlet state excitation by light absorption, then inter-system crossing to the triplet state, and finally reduction by DTT oxidation to DTTc. At pH > 6.7 the reaction reads



(R3)

Air injection to the photo-reduced sample led to prompt re-oxidation of $FMN_{red}H^-$ to FMN_{ox}. The formed DTTc is no oxygen scavenger.

The efficiency of photo-reduction of FMN in pH 8 buffer with 1 mM EDTA and 10 mM DTT is approximately the same as the efficiency of photo-reduction of DTT alone. The oxygen scavenging action of the EDTA photoproducts remains in the EDTA and DTT reducing agent mixture.

4.2. Aerobic photo-reduction

Under aerobic conditions in the absence of exogenous reducing agents, the photo-degradation of FMN dominates over the photo-reduction of FMN in pH 8 buffer. The fast re-oxidation of reduced FMN in the presence of dissolved oxygen makes the formed fraction of reduced FMN smaller than the fraction of photoproducts. The quantum yield of photo-degradation was determined to be $\phi_D \approx 4.6 \times 10^{-3}$ [35] while the quantum yield of photo-reduction under anaerobic conditions was found here to be $\phi_{red,0} \approx 0.022$. Photo-excitation of FMN ($^1FMN + h\nu \rightarrow {}^1FMN^*$) causes triplet formation ($^1FMN^* \rightarrow {}^3FMN$), and collision of triplet FMN with ground-state triplet oxygen ($^3O_2$, $^3\Sigma_g^-$) causes singlet oxygen generation ($^1O_2$, $^1\Delta_g$) by Dexter-type excitation transfer [46]. The generated $^1O_2$ is consumed by chemical reaction of the singlet oxygen (photo-degradation of FMN, and reaction with the solvent like $^1O_2 + 2H_2O \rightarrow 2H_2O_2$). After oxygen consumption some permanent FMN reduction occurs. This fraction of reduced FMN could be determined by air injection after the excitation process showing up in an absorption step (re-oxidation of reduced FMN).

The photo-reduction of FMN in the presence of 1 mM EDTA starts slightly time-delayed (only small absorption decrease within the first 30 s of exposure). The dissolved oxygen in water (approximately 2.8×10^{-4} mol dm^{-3} at room temperature [34]) delays the photo-reduction of FMN. The maximum quantum yield of photo-reduction ($\phi_{red,max} \approx 0.072$) is reduced compared to de-aerated solution ($\phi_{red,0} \approx 0.15$) likely by EDTA consumption by reaction with generated singlet oxygen. During photo-excitation the dissolved oxygen is consumed up (no prompt re-oxidation after light switch-off). Some photoproduct of EDTA formed by photo-reduction of FMN acts as oxygen scavenger leading to rather slow re-oxidation of reduced FMN due to air in-diffusion. The reduced flavin $FMN_{red}H_2$ re-oxidizes practically completely back to the original oxidized flavin FMN_{ox}. The irradiation products from the EDTA oxidation were found to be carbon dioxide, an amine residue, and formaldehyde [17,27].

The photo-reduction of FMN under aerobic conditions in the presence of 10 mM DTT is slightly time delayed (only small absorption decrease within the first 30 s of light exposure) as in the case of EDTA presence. After the initial oxygen consumption the photo-reduction under aerobic conditions in the presence of 10 mM DTT is nearly as effective as under anaerobic conditions. The concentration of DTT is roughly a factor of 40 higher than the dissolved oxygen concentration, so the fraction of DTT consumed by reaction with singlet oxygen is small compared to the total DTT concentration. The dissolved oxygen is consumed up by FMN photo-excitation. After light switch-off the re-oxidation starts without delay with a time of recovery of $t_{rec,1/e} \approx 8.5$ min (no scavenging effect). The speed of re-oxidation is determined by the in-diffusion of air into the sample. Practically a complete re-oxidation is observed. Air-infusion after light switch-off causes a prompt complete re-oxidation.

The photo-reduction of FMN under aerobic conditions in the presence of 1 mM EDTA and 10 mM DDT is less efficient than the photo-reduction of EDTA alone. The onset of photo-reduction is more delayed and the speed of re-oxidation after light switch-off is slower than in the case of application of EDTA alone. The effects of EDTA and DTT are not simple additive. Some photochemical interaction between EDTA and DTT seems to take place in the presence of FMN.

4.3. Photo-reduction of other flavins in aqueous solution

The photo-reduction of flavins in aqueous solution is not restricted to FMN [21,22,25,28,42–44]. We performed preliminary photo-reduction studies on riboflavin, flavin adenine dinucleotide (FAD), and lumiflavin in aqueous pH 8 buffer solution with added 1 mM EDTA and 10 mM DTT. Thereby we observed similar permanent photo-reduction under anaerobic conditions, and similar reversible photo-reduction under aerobic conditions.

4.4. Photo-reduction of flavins in photoreceptors

The fluorescence and optical characteristics of some reduced flavines and flavoproteins was studied in [14].

In DNA photolyases the cofactors are MTHF (*N*5,*N*10-methenyl-5,6,7,8-tetrahydrofolate) and FAD. In the dark FAD is present in its anionic reduced state ($FAD_{red}H^-$), and the photolyase photo-cycle leads to neutral FAD semiquinone formation ($FADH^{\cdot}$) as an intermediate [47].

Till now three classes of blue-light photoreceptors with flavin cofactors have been discovered, the phototropins with LOV domains (LOV = *l*ight, *o*xygen, *v*oltage, cofactor

FMN), the BLUF receptors (BLUF = *b*lue-*l*ight receptors *u*sing *F*AD, cofactor FAD), and the cryptochromes (cofactor FAD) [3–5,48].

In the cryptochromes cry1 from *Arabidopsis thaliana* [49] and in cry2 from *Arabidopsis thaliana* (A. Batschauer, private communication) the dark-state FAD_{ox} is photo-reduced in the photo-cycle to the intermediate neutral semi-reduced $FADH^{\bullet}$. In cry3 from *Arabidopsis thaliana* the cofactors FAD_{ox}, $FADH^{\bullet}$, and $FAD_{red}H_2$ or $FAD_{red}H^-$ are present in the dark together with MTHF [50]. FAD_{ox} as well as $FADH^{\bullet}$ are reversibly photo-reduced to $FAD_{red}H_2$ or $FAD_{red}H^-$ [50]. For dcry from *Drosophila melanogaster* blue-light photo-excitation causes reversible reduction of FAD_{ox} to the anionic semiquinone form $FAD^{\bullet -}$ (E. Wolf, private communication, local pH likely higher than 8.3 [1]).

In the BLUF domain BlrB from *Rhodobacter sphaeroides* [51] reversible FAD hydroquinone formation via semiquinone formation from the photo-excited signalling state was observed by prolonged blue-light exposure [52]. In the BLUF domain Slr1694 from *Synechocystis* pc. PCC6803 [53] reversible flavin hydroquinone formation from the photo-excited signalling state was found by prolonged blue-light exposure [54].

In LOV domains of phototropin photoreceptors [55,56] the photo-reduction of the cofactor FMN_{ox} is hindered by efficient FMN-C(4a)-cysteinyl adduct formation [55,56]. In some mutants of LOV domains (active Cys residue replaced by another amino acid residue) where FMN cysteinyl adduct formation is impossible, photo-reduction to the neutral semiquinone form was observed under aerobic and anaerobic conditions with and without the addition of exogenous electron donors [57–59]. The semiquinone form is stable since disproportionation of two flavosemiquinones to a flavoquinone and a flavohydroquinone is hindered by the flavin protection in the protein binding pocket.

5. Conclusions

The photo-reduction of FMN (concentration around 0.15 mM) in aqueous solution at pH 8 was studied under aerobic and anaerobic conditions. Under anaerobic conditions the reduction remains permanent while under aerobic conditions re-oxidation on a minute timescale takes place. The reduction is thought to occur from the triplet state of the excited molecules. The efficiency of photo-reduction of FMN without an added reducing agent was found to be in the two percent range under anaerobic conditions, and under aerobic conditions the photo-degradation dominated. The efficiency of photo-reduction is reasonably high in the presence of 1 mM EDTA under anaerobic conditions (≈15%), and is approximately a factor of two lower under aerobic condition. Similarly efficient photo-reduction (≈8%) of FMN under anaerobic and aerobic conditions was observed in the presence of DTT.

In the process of flavin photo-reduction under aerobic conditions, both with and without the application of a reducing agent, the dissolved oxygen is consumed due to singlet oxygen generation by de-activation of triplet excited flavin and subsequent chemical singlet oxygen reaction with solvent and solute. The photo-reduction of FMN in the presence of EDTA causes EDTA destruction with the formation of an oxygen scavenging product (chemical oxygen getter).

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