

Time-resolved and steady-state fluorescence measurements of β -nicotinamide adenine dinucleotide–alcohol dehydrogenase complex during UVA exposure

Karsten König^{a,c}, Michael W. Berns^b, Bruce J. Tromberg^c

^a Institute of Anatomy II, Friedrich Schiller University, D-07743 Jena, Germany

^b Institute for Molecular Biotechnology, D-07708 Jena, Germany

^c Beckman Laser Institute and Medical Clinic, Irvine, CA 92715, USA

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Abstract

β -nicotinamide adenine dinucleotide (NADH)–alcohol dehydrogenase complex was exposed to either UVA irradiation (364 nm; 50 mW cm⁻²; 0–60 min) or heat in order to investigate complex stability. Prior to irradiation, frequency-domain fluorescence lifetime measurements indicated the presence of two principal components having short (subnanosecond) and long (nanosecond) fluorescence lifetimes reflecting free and bound NADH respectively. UVA exposure resulted in decreased NADH fluorescence intensity concomitant with decreased absorption at 337 nm. However, UVA irradiation did not reduce the fractional contribution of the long-lived bound NADH. The photoinduced fluorescence decrease appeared to be caused by the formation of oxidized NAD⁺ and not on UV-induced dissociation of the NADH–protein complex. Such dissociation, detected by a red-shifted fluorescence maximum and decreased fractional contribution of the nanosecond component, was observed when NADH–protein mixtures were heated.

Keywords: NADH; Photo-oxidation; UVA; Time-resolved fluorescence

1. Introduction

Cellular autofluorescence in the visible spectral region provides information on metabolism and intracellular redox state. Major endogenous fluorophores are the reduced pyridine coenzymes β -nicotinamide adenine dinucleotide (NADH), and β -nicotinamide adenine dinucleotide phosphate (NADPH) as well as oxidized flavin coenzymes.

NAD(H) acts as hydrogen-transferring molecule in the respiratory chain. The hydrogen uptake at position 4 of the planar pyridine ring is responsible for significant spectral differences between reduced and oxidized form. In contrast with oxidized NAD⁺, the reduced coenzyme NADH absorbs strongly around 340 nm and fluoresces in the blue spectral region with a 440–460 nm fluorescence maximum [1]. The mean fluorescence lifetime of NADH depends on solvent and temperature and was found to be in the subnanosecond region (e.g. [2–5]). Monoexponential, biexponential and triexponential fitting of the fluorescence decay of NADH in various solvents has been reported [2–5].

NADH serves as coenzyme for hundreds of different dehydrogenases. It exhibits an extended conformation when bound to an enzyme [1,3]. The binding to an apoprotein of a dehydrogenase results in a blue-shifted fluorescence maximum [1,6], increased nanosecond fluorescence lifetimes [2] and therefore enhanced fluorescence intensity.

In recent studies [7] we found significant changes in intracellular autofluorescence during UVA exposure (365 nm). An intensity decrease at 450 nm to 30% of the value prior to irradiation was detected for radiant exposures up to 160 J cm⁻². Further UVA exposure (radiant exposures, greater than 160 J cm⁻²) resulted in immediate cell death. Cells were no longer able to exclude the dead cell indicator Trypan Blue and showed autofluorescence increase [7].

UV-induced decrease of NADH fluorescence was also detected by Veshkin [8] in NADH–alcohol dehydrogenase (ADH) complex using steady-state fluorescence spectroscopy. He suggested UV-induced transformation of bound NADH to free NADH by desorption.

It was our goal to study the binding properties of the NADH–ADH complex during light (UVA) or heat exposure by time-resolved and steady-state fluorescence spectroscopy.

2. Materials and methods

ADH (formula weight, 141.000) from Bakers Yeast was purchased from Sigma and diluted with calcium- and magnesium-free phosphate buffer saline (PBS) (pH 7.4). NADH (formula weight, 781) was obtained in pre-weighed glass vials (Sigma) and dissolved in PBS (pH 7.4) 2 h prior to experiment. The final NADH concentration was $25.6 \mu\text{M}$ ($20 \mu\text{g ml}^{-1}$), of not otherwise mentioned. Samples (2 ml) were placed in a 1 cm quartz cuvette and stirred. The temperature of the solutions was permanently monitored using a copper–constantan thermocouple. Samples were heated with a thermostat; the heating speed was $3 \text{ }^\circ\text{C min}^{-1}$. Room temperature was $25 \text{ }^\circ\text{C}$. During UVA exposure, temperature variations less than $0.5 \text{ }^\circ\text{C}$ in the NADH solutions were measured.

The 363.8 nm radiation of a continuous-wave Ar^+ laser served as UVA source for exposure of the NADH–protein mixture. The solution was irradiated with an intensity of 50 mW cm^{-2} up to 60 min (irradiation spot diameter, 1 cm; radiant exposure, 180 J cm^{-2} or less). The time interval between UVA exposure and beginning of the fluorescence measurements was 50 s.

Steady-state as well as time-resolved fluorescence measurements were carried out with a Fourier transform spectrofluorometer SLM 48000 MHF (SLM Aminco) with picosecond time resolution. A 450 W xenon arc lamp was used for steady-state measurements. Radiation at 337 nm (selected with a single-grating monochromator with 4 nm slits) provided the excitation of steady-state NADH fluorescence. Time-resolved measurements were performed in the frequency domain where intensity-modulated fluorescence excitation is required. For that purpose, the 363.8 nm beam was transmitted to a Pockel cell operating up to 250 MHz, resulting in modulation with multiple sinusoidal Fourier components. The fluorescence signal is also modulated but phase delayed and demodulated owing to the specific fluorescence lifetimes of the fluorophores. For lifetime determination, phase shifts and demodulation at different frequencies have to be recorded. In our experiments, each frequency-domain lifetime measurement consisted of an average of 200 recordings at 50 frequencies (5–250 MHz) which, in multiharmonic mode, required approximately 30 s. In order to avoid systematic errors due to color effects, 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) in ethanol with a fluorescence maximum at 420 nm and a single lifetime of 1.29 ns [5] was used as reference. Fluorescence decay times and fractional contributions were determined from phase shift and demodulation data by the non-linear least-squares methods assuming a multiexponential fluorescence decay (SLM software, version 1.2). Owing to the low signal-to-noise ratio for high

frequencies, fitting of phase shift and demodulation was performed up to 200 MHz. Absorption spectra were measured with a DU-7 Beckman single-beam spectrophotometer. A PBS-filled 1 cm quartz cuvette served as reference.

3. Results

3.1. Spectral and temporal characteristics prior to UVA exposure

The absorption spectrum of $25.6 \mu\text{M}$ NADH in PBS (pH 7.4) showed maxima at 337 and 259 nm with an intensity ratio of 1:2.5. The 259 nm band of free NADH is due to adenine absorption, whereas the 337 nm electronic transition is characteristic of the reduced state of the coenzyme (dihydronicotinamide system). At the 364 nm laser wavelength the molar extinction coefficient ($\epsilon = 3.9 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$) was about half the maximum value. NADH–ADH mixtures ($25.6 \mu\text{M}$ NADH; $5 \mu\text{M}$ ADH) exhibited maxima at 337 nm, 277 nm and 228 nm with an intensity ratio of 1:7.5:15, indicating the additional absorption by amino acids in the shorter-wavelength region. The optical density (OD) at 337 nm was the same as for free $25.6 \mu\text{M}$ NADH.

Free NADH fluoresced with a 455 nm peak, whereas the NADH–ADH complex resulted in a blue-shifted fluorescence maximum at 445 nm and an increased NADH fluorescence quantum yield (Fig. 1). It was found that the fluorescence decay of free NADH could be fitted as nearly monoexponential with a mean lifetime $\tau = 300 \pm 50 \text{ ps}$. In contrast, NADH–ADH showed biexponential fluorescence kinetics with an additional lifetime of about $2.9 \pm 0.2 \text{ ns}$ for protein-bound NADH. We measured the fractional contributions I_1 (picosecond component) and I_2 (nanosecond component) of NADH–ADH mixtures ($25.6 \mu\text{M}$ NADH) in the presence of varying amounts of the protein ADH. Values of $I_1 = 100\%$ ($I_2 = 0\%$), 77% (23%) and 41% (59%) were determined for ADH concentrations of $0 \mu\text{M}$, $23 \mu\text{M}$ and $59 \mu\text{M}$ respectively.

3.2. UV exposure

UV exposure of free NADH resulted in decreased fluorescence intensity as well as in reduced OD at 337 nm (Fig. 2).

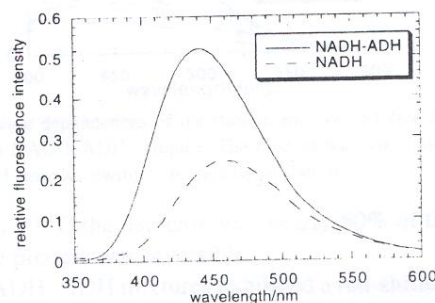


Fig. 1. Fluorescence spectrum of $25.6 \mu\text{M}$ NADH and NADH–ADH complex ($5 \mu\text{M}$ ADH) in PBS.

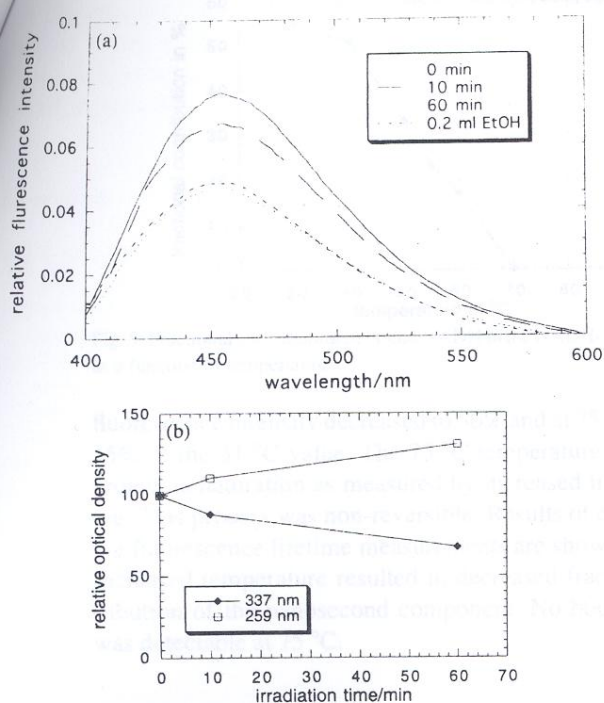


Fig. 2. UVA-induced changes in (a) the fluorescence and (b) the absorption of free NADH (25.6 μM). Ethanol was added after UVA exposure.

As shown, a radiant exposure of 180 J cm^{-2} (60 min) was necessary to decrease the OD(337 nm) to about 70% of the initial value. Interestingly, the absorption peak at 259 nm increased slowly with increasing UV exposure (130% after 60 min irradiation). No UVA-induced change in the fluorescence lifetime was observed.

In contrast, UV exposure to NADH-ADH mixtures (25.6 μM NADH; 10 μM ADH) led to a faster decrease in fluorescence as well as in 337 nm absorbance (Fig. 3). The absorbance increase at the short-wavelength band (227 nm) was less significant owing to the superposition of coenzyme and enzyme absorption. No decrease in the fractional contributions of the two fluorescent components nor a NADH fluorescence maximum shift were found.

Ethanol (0.2 ml) was added to the solution irradiated for 60 min to see whether the UV-induced OD decrease at 337 nm and the OD increase at 277 nm indicated NAD^+ formation. Ethanol leads to NAD^+ reduction and stable NADH-ADH complex formation via ADH-catalyzed reaction [1,8]. We found indeed a nearly twofold absorbance increase at 337 nm concomitant with a twofold fluorescence increase at 445 nm after incubation with ethanol (Fig. 3). The 277 nm absorbance decrease was found to be small, probably owing to strong protein absorption and the sample dilution.

3.3. Temperature dependence

The fluorescence dependence on temperature for free NADH solution is shown in Fig. 4(a). With enhanced temperature the intensity of the 455 nm fluorescence maximum

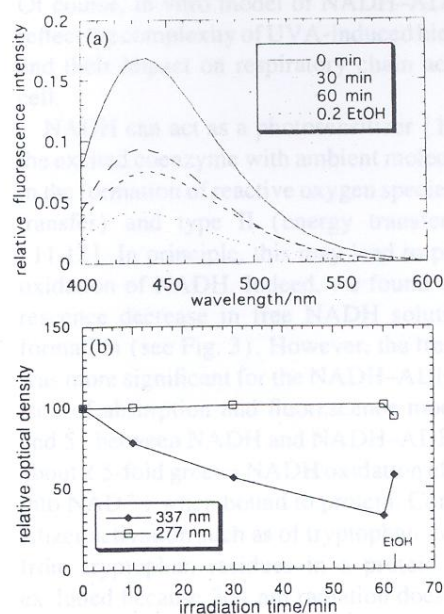


Fig. 3. UVA-induced changes in (a) the fluorescence and (b) the absorption of bound NADH (25.6 μM NADH; 10 μM ADH). Ethanol was added to the solution irradiated for 60 min. In the presence of ADH, UVA-induced NAD^+ is transformed into a stable NADH-ADH complex.

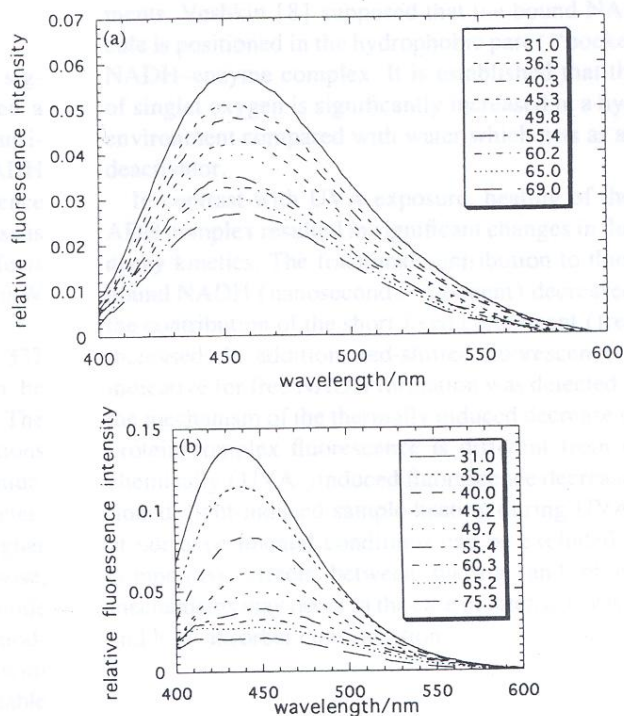


Fig. 4. Temperature dependences of the fluorescence of (a) free NADH solution and (b) NADH-ADH complex. The process was irreversible for the NADH-ADH complex owing to protein denaturation.

decreased. At 69 $^{\circ}\text{C}$ the decrease was nearly 50% of the 31 $^{\circ}\text{C}$ value. The process was reversible.

Heated NADH-ADH mixtures exhibited a red-shifted fluorescence maximum, indicating the transition into free NADH (Fig. 4(b)). A higher fading rates as in the case of free NADH was measured. At about 45 $^{\circ}\text{C}$ the maximum

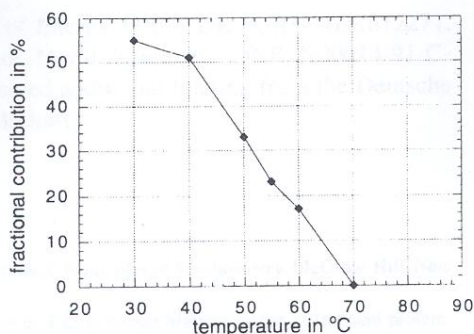


Fig. 5. Fractional contribution of bound NADH in the NADH-ADH mixture as a function of temperature.

fluorescence intensity decreased to 50% and at 75 °C to about 15% of the 31 °C value. The 75 °C temperature resulted in protein denaturation as measured by increased light scattering. This process was non-reversible. Results of corresponding fluorescence lifetime measurements are shown in Fig. 5. Increased temperature resulted in decreased fractional contribution of the nanosecond component. No bound NADH was detectable at 75 °C.

4. Discussion

UVA exposure to free and bound NADH resulted in significant fluorescence decrease. Bound NADH exhibited a higher fading rate. No evidence for an UVA-induced transition of protein-bound NADH into short-lived free NADH was found (no fluorescence shift, no change in fluorescence decay kinetics). The UVA-induced fluorescence decrease is attributed to photochemical reactions. Temperature effects can be excluded at our experimental conditions of 50 mW cm⁻² UVA intensity and 0.1 OD.

We detected an UVA-induced absorption decrease at 337 nm and an increase in the 259 nm band which can be explained with formation of the oxidized form NAD⁺. The 250 nm absorption band of NAD⁺ contains contributions from two chromophores, namely adenine and nicotinamide, in contrast with NADH where the absorption band is determined by adenine only [10]. NAD⁺ has therefore a higher 259 nm absorption coefficient than NADH [10]. Otherwise, the 337 nm band is due entirely to the dihydronicotinamide system (NADH) [10]. The UVA-induced absorption modification resulted in fluorescence decrease. In contrast with NADH, the fluorescence from NAD⁺ was not detectable because of an extremely low fluorescence quantum yield. A further indication for UVA-induced NAD⁺ formation was given by the enhanced NADH fluorescence after ethanol incubation of irradiated NADH-ADH solutions.

We conclude that NADH photo-oxidation and transition into non-fluorescent NAD⁺ is responsible for fluorescence modifications of NADH-protein complexes in solution. This photo-oxidation could also be the reason for the observed decrease in cellular autofluorescence during UVA exposure.

Of course, in vitro model of NADH-ADH complex cannot reflect the complexity of UVA-induced biochemical reactions and their impact on respiratory chain activity in the living cell.

NADH can act as a photosensitizer [11,12]. Reaction of the excited coenzyme with ambient molecular oxygen results in the formation of reactive oxygen species via type I (charge transfer) and type II (energy transfer) photo-oxidation [11,12]. In principle, this may lead to photo-induced auto-oxidation of NADH. Indeed, we found UVA-induced fluorescence decrease in free NADH solution due to NAD⁺ formation (see Fig. 3). However, the transition into NAD⁺ was more significant for the NADH-ADH mixture. Comparison of absorption and fluorescence modifications (Figs. 4 and 5) between NADH and NADH-ADH complex revealed about 2.5-fold greater NADH oxidation efficiency (transition into NAD⁺) when bound to protein. Competitive photosensitizer activation such as of tryptophan, or an energy transfer from tryptophan residues in a protein to NADH can be excluded because 364 nm radiation does not excite proteins efficiently (no UVA absorption maxima). One possible explanation for the efficient transition of bound NADH into NAD⁺ may be increased rates of singlet oxygen formation and singlet oxygen lifetimes due to different microenvironments. Veshkin [8] supposed that the bound NADH molecule is positioned in the hydrophobic part ("pocket") of the NADH-enzyme complex. It is established that the lifetime of singlet oxygen is significantly increased in a hydrophobic environment compared with water which acts as an efficient deactivator.

In contrast with UVA exposure, heating of the NADH-ADH complex resulted in significant changes in fluorescence decay kinetics. The fractional contribution to fluorescent of bound NADH (nanosecond component) decreased, whereas the contribution of the short-lived component (free NADH) increased. In addition, red-shifted fluorescence maximum indicative for free NADH formation was detected. Therefore the mechanism of the thermally induced decrease in NADH-protein complex fluorescence is different from the photochemically (UVA-)induced fluorescence decrease. As mentioned, light-induced sample heating during UVA exposure at our experimental conditions can be excluded. However, competitive effects between thermal and photochemical mechanisms may occur in the case of intense UVA irradiation and high absorber concentration.

Acknowledgments

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