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Temperature Dependence for Fluorescence of β-NADH in Glycerol/Water Solution and in Trehalose/Sucrose Glass

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Abstract Fluorescence imaging of cells and tissue can be used to evaluate β-NADH redox and location. At low temperature, β-NADH fluorescence intensity increases and therefore sensitivity of imaging increases. In this paper, the temperature dependence of fluorescence was evaluated for β-NADH in glycerol/water solution and in trehalose/sucrose glass. The average fluorescence lifetime for NADH in glycerol/water is 0.66 ns, compared with 5.3 ns in trehalose/sucrose at 20°C. Emission spectra were recorded from 290 to 12 K. The fluorescence of β-NADH in glycerol/water increases ∼16 fold and the emission shifts about 35 nm to the blue as temperature decreases. Much smaller change is seen for fluorescence of β-NADH in sugar glass. Below 77 K, the β-NADH spectral features did not change significantly with temperature change, and so no increase in sensitivity is obtained by going to very low temperatures. It is suggested that the sensitivity of β-NADH fluorescence is related to water relaxation around the excited state molecule. Differences in water in various tissues may contribute to β-NADH fluorescence changes when cells are altered.

Keywords NADH · Sugar glass · Cryogenic · Trehalose · Bioprotectant

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Introduction

Nicotinamide adenine dinucleotide is highly fluorescent in the reduced form, (NADH), but is non-fluorescent when oxidized (NAD). The fluorescence of β-NADH has been widely exploited to evaluate redox conditions in tissues and cells [1] and for studying binding of β-NADH to enzyme dehydrogenases [2]. β-NADH along with tryptophan, tyrosine and porphyrin are the most highly used intrinsic fluorescent probes of biological systems.

When combined with imaging, fluorescence of β-NADH gives information both on redox state and its spatial distribution in tissue and cells [3–5]. An application of NADH fluorescence imaging is in freeze-trap experiments of tissue or cells. Lowering the temperature inhibits metabolism, and allows the study of tissue in stable conditions [6]. An added advantage of freeze-trap experiments is that at low temperature the fluorescence intensity of β-NADH increases, leading to increased sensitivity [7].

In this paper, we are asking how the fluorescence spectrum and intensity changes with temperature. Two conditions are used to study β-NADH fluorescence as a function of temperature. For one condition, the solvent is glycerol/water, a solvent that is widely used experimentally as a cryoprotectant [8]. The other condition uses a sugar glass matrix. The disaccharide trehalose is used by many organisms to protect against extreme temperature and dehydration [9]. In a sugar matrix composed of trehalose and sucrose, the glass is stable and water can be mostly eliminated, while proteins maintain structure [10, 11]. Both glycerol/water and sugar glass represent media that are rich in OH groups but in case of glycerol/water, the sample is a liquid at high temperature, and glassifies at low temperature. In contrast, the sugar matrix is solid over the entire temperature range studied. Comparison of the two matrices allows us to evaluate the

role of water rearrangement on β-NADH fluorescence. The importance of relaxation of water is exemplified by Trp fluorescence. In glycerol/water Trp emission spectrum is very much dependent upon temperature. But in sugar glass matrix no large shift is seen [12]. If β-NADH undergoes a similar water-dependent shift, the conclusion would be that the emission of β-NADH, like Trp, shifts with the relaxation of water molecules around the excited state molecule.

The results have practical application for the use of β-NADH fluorescence to study cells. In order to optimize measuring conditions, excitation and emission wavelengths should be judiciously chosen, and the temperature dependence will also reveal the optimum temperature for measurement. With temperature change, there is a lifetime change, another parameter that can be used to increase the sensitivity and specificity of detection [13].

Experimental

Materials

Water was deionized and glass distilled. Glycerol 99%, α-D-glucopyranosyl-β-D-glucopyranoside (D(+) trehalose), α-D-glucopyranosyl-β-D-fructofuranoside (sucrose), and β-nicotinamide adenine dinucleotide, reduced, sodium salt (β-NADH) were supplied by Sigma Chemical Co. (St. Louis, MO).

Sample preparation

Optically clear trehalose/sucrose glass was prepared as previously described [11]. To prepare the sugar glass sample, 0.5 mg β-NADH was dissolved in 350 ml of water and 150 ml of stock sugar solution (300 mg trehalose and 300 mg sucrose dissolved in 500 μl water). The solution was placed on a 25 mm round quartz plate of 1 mm thickness (Esco Products, Oak Ridge, NJ). The quartz disk and sample was placed on a VWR Scientific Products Heat Block at 65 to 70°C for 2 h until dry.

The concentration of β-NADH in the 60/40% glycerol/water was 1 mg/ml. The solution was placed between two circular quartz plates with a 200 μm Teflon spacer.

Steady-state fluorescence

Emission spectra were measured with a Fluorolog-3-21 Jobin-Yvon Spex Instrument SA (Edison, NJ) equipped with a 450 W Xenon lamp for excitation and a cooled R2658P Hamamatsu photomultiplier tube for detection. Front-face geometry was used for all temperature-dependent fluorescence measurements. Excitation wavelength was 340 nm. Slit width was set to provide a band-pass of 2 nm for excitation and 1 nm for emission.

Sample temperature for temperature-dependent emission measurements was regulated using an APD closed cycle Helitran cryostat (Advanced Research Systems, Allentown, PA). A chamber was constructed to alleviate strain on windows due to contraction at low temperature. For fluorescence/phosphorescence measurements, the outer windows of the compartment were made of quartz and the inner windows were made of sapphire. Temperature was measured and controlled using a silicon diode near the sample connected to a Model 9650 temperature controller (Scientific Instruments, Palm Beach, FL). Temperature dependent measurements were performed from high to low temperature at 10 K increments. OPUS program (Bruker Optics) was used to display the spectra and to determine the maxima of emission.

Time-resolved fluorescence

Fluorescence decay traces were measured at the Regional Laser and Biomedical Technology Laboratories (RLBL) of the University of Pennsylvania. The frequency-doubled output from a Nd:YAG pumped rhodamine 6G dye laser at 280 nm was used for excitation. Measurements were taken at ambient temperature. Fluorescence was detected by a MCP photomultiplier (Hamamatsu R3809U) at magic angle and through a subtractive double monochromator. Decay traces were recorded with a Becker-Hickl PCI TCSPC board SPC-730 and the corresponding software, and analyzed with deconvolution using the FluoFit software package from PicoQuant GmbH.

UV/vis absorption

A Hitachi Perkin-Elmer U-3000 absorption instrument (Hitachi Instruments, Danbury CT) was used to take the UV - visible absorption spectra.

Results

Spectra of β-NADH

Figure 1 shows excitation and emission spectra of β-NADH in water at ambient temperature and in glycerol/water at 12 K. The excitation spectrum is shifted slightly with a slight narrowing of the band at low temperature. Temperature-dependent changes in the absorption of β-NADH are known for the temperature range of 0–38°C [14]. The change in the emission spectrum with temperature is larger than for absorbance. At 290 K the emission maximum is at 449 nm. At low temperature the maximum is at 415 nm. Emission spectra of β-NADH in water, methanol and ethanol solutions were also compared (spectra not shown). At 300 K, the emission maxima are 454, 448, 442 nm, respectively.

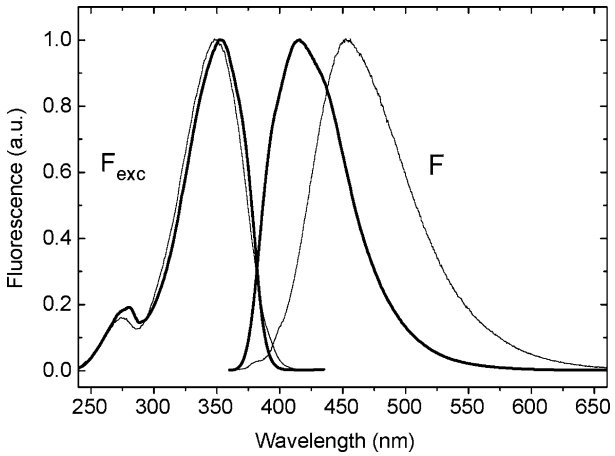

Fig. 1 Fluorescence excitation (F_{ex}, λ_{em} = 450 nm) and fluorescence emission (F, λ_{exc} = 340 nm) spectra of β-NADH in water at 295 K (*thin lines*) and in glycerol/water (60/40, v/v) at 12 K (*thick lines*)

Emission of β-NADH was examined over the temperature range of 290–12 K. Emission spectra of β-NADH in glycerol/water or trehalose/sucrose glass are shown in Figs. 2 and 3, respectively. In these Figures, the intensity of emission at 290 K was normalized to 1; in this way

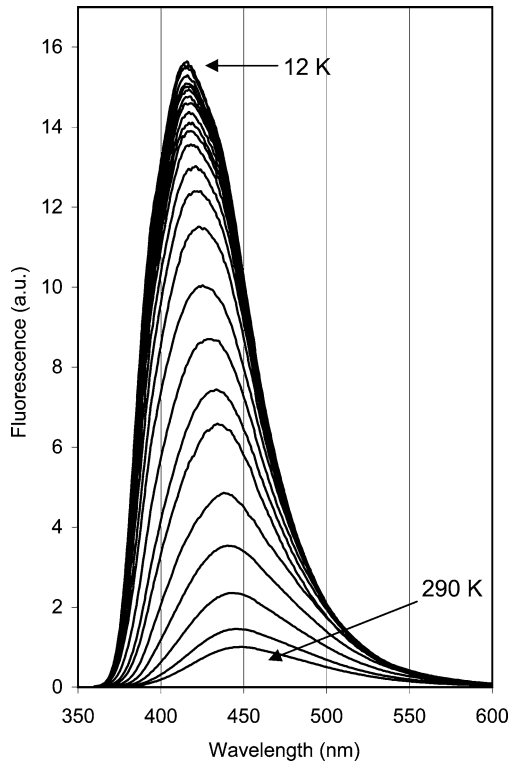

Fig. 2 Fluorescence emission of β-NADH in glycerol/water (60/40 v/v). Temperatures at 12 and 290 K are indicated. Other temperatures from low intensity to high are 270–90 K in 10° increments, and then 70, 50, 30 and 12 K

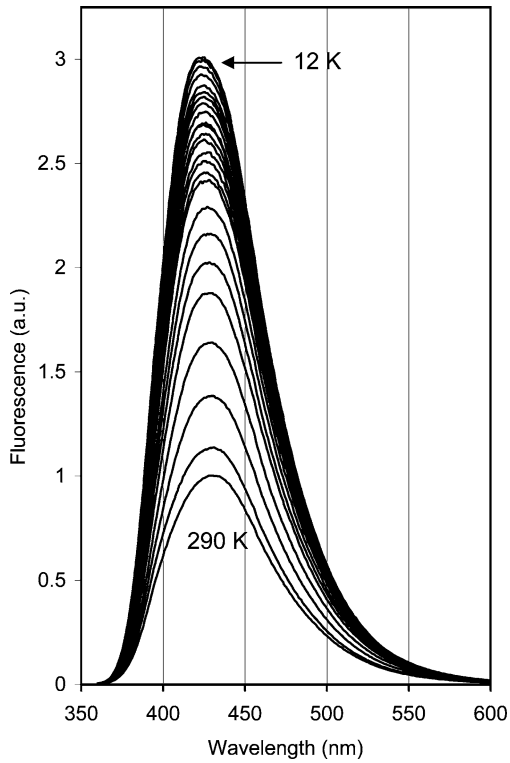

Fig. 3 Fluorescence emission of β-NADH in trehalose/sucrose glass. Temperatures at 12 and 290 K are indicated. Other temperatures from low intensity to high are 270 to 90 K in 10° increments, and then 70, 50, 30 and 12 K

the *y*-axis shows the factor for increase in fluorescence as temperature is lowered. The spectral changes reversed when the temperature was brought back to ambient temperature.

β-NADH fluorescence features as a function of temperature

The shift in the spectra (seen in Figs. 2 and 3) with temperature is greater for β-NADH in glycerol/water than in trehalose/sugar glass. The emission maxima are presented in Fig. 4. The fluorescence of β-NADH in the nearly anhydrous sugar glass is blue shifted at room temperature, and only shifts about 6 nm further blue as temperature decreases. In contrast, the fluorescence of β-NADH in glycerol/water shifts about 35 nm over the temperature excursion.

The temperature dependence for the intensity is shown in Fig. 5. As temperature is lowered the intensity at the emission maximum increases 3 times for β-NADH in trehalose/sucrose glass and nearly 16 times in glycerol/water. Because of the spectral shift for the fluorescence of β-NADH in glycerol/water, the intensity change is greater on the blue edge. At 400 nm, for instance, the increase in yield with decreasing temperature is nearly 30 times.

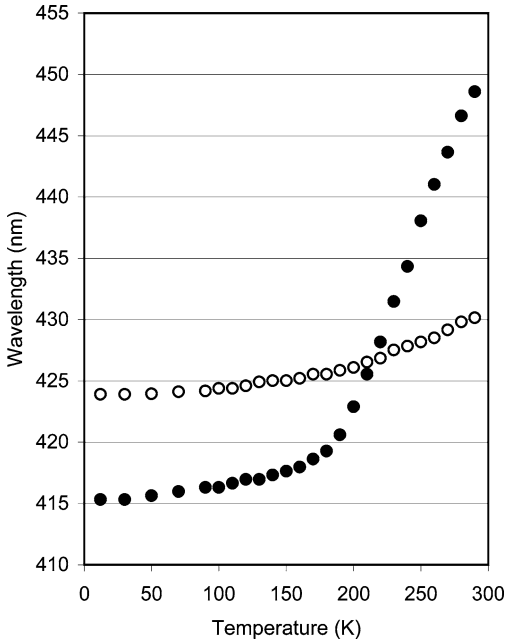

Fig. 4 Fluorescence emission maxima of β-NADH as a function of temperature. Closed circles: β-NADH in glycerol/water (60/40 v/v); data from Fig. 2. Open circles: β-NADH in trehalose/sucrose glass, data from Fig. 3

Fluorescence lifetimes in glycerol and in sugar glass

The fluorescence decay profiles of β-NADH in glycerol/water and trehalose/sucrose glass are given in Fig. 6, and fit parameters are given in Table 1. The lifetime found for

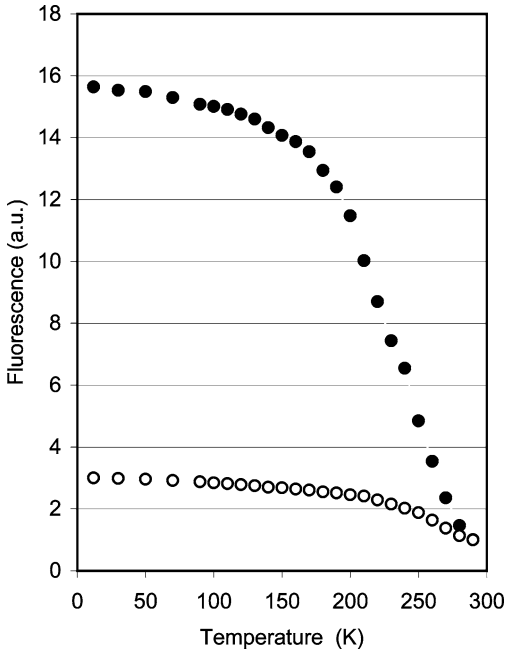

Fig. 5 Emission intensity of β-NADH as a function of temperature. Closed circles: data for β-NADH in glycerol/water, data from Fig. 2. Open circles: β-NADH in trehalose/sucrose glass, data from Fig. 3

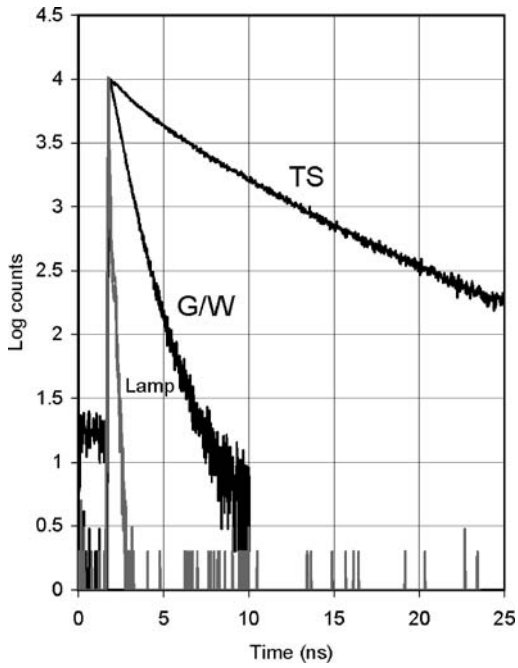

Fig. 6 Fluorescence emission of β-NADH in TS (trehalose/sucrose glass), G/W (glycerol/water, 60/40). Fit parameters are given in Table 1

β-NADH in glycerol/water at 20°C is close to that found for β-NADH in water (Table 1 and [13]). The long-lived component of fluorescence decay is $\sim$6 times greater for β-NADH in trehalose/sucrose glass as in liquid glycerol/water. The chemical environments of sugar glasses and glycerol/water solution are very similar, but differ physically in the sense that the former is solid and glycerol/water is liquid.

Discussion

The fluorescence yield and spectrum of β-NADH in glycerol/water is dependent upon temperature. Lowering temperature increases the fluorescence intensity, and shifts the spectrum to the blue. These effects are much larger for β-NADH in glycerol/water compared to trehalose/sucrose glass.

Table 1 Fluorescence lifetime of β-NADH at 20°C

Solvent or matrix	τ_i, nsec	α_i	$<\tau_i>$, nsec	X_R^2
Water	0.16	0.49	0.52	1.27
	0.48	0.50		
	2.4	0.09		
Glycerol/water	0.04	0.53	0.66	1.89
60/40 (v/v)	0.48	0.37		
	1.07	0.10		
Trehalose/sucrose	0.08	0.37	5.28	1.15
glass	1.9	0.27		
	6.1	0.36		

In general, molecules that experience spectral shifts with temperature change have a large change in the dipole moment between ground and excited states [15, 16]. As dipolar solvent molecules rearrange around the molecule in the excited state, the energy level is lowered, and the molecule exhibits a red shift in the emission. The extent of the red shift is a function of the difference in dipole moment of the ground and excited state molecule, the dipole moment of the solvent molecules, and the ability of the solvent molecules to rearrange. In the solvent series water, methanol and ethanol, the emission is the most blue shifted in ethanol solution, a sequence that is observed for molecules that have a large dipole change in the excited state. As temperature decreases, the solvent water molecules in the aqueous glycerol solution can no longer rearrange within the time scale of the excited state. In this case the spectrum also becomes blue shifted. The shift of the emission maximum for β-NADH is large: $\sim$35 nm over the temperature range (Figs. 1 and 4). The shift in the spectrum for β-NADH fluorescence occurs at the same temperature range where H-bonding increases between water and glycerol molecules [17].

The fluorescence of β-NADH in the trehalose/sucrose glass shows much less temperature dependence in comparison to glycerol/water. The intensity increases only about 3 times as temperature decreases, and the spectral maximum shifts only 5–6 nm. The longer lifetime at room temperature (5.28 ns in comparison with 0.66 ns in glycerolwater) and the lack of change in the emission maximum is consistent with the view that the solvent cannot rearrange in the lifetime of the excited state for this solid matrix. It is note-worthy that the sugar glass maintains the room temperature parameters of β-NADH so well at low temperature. Trehalose is a sugar that maintains cells under extreme temperature and dehydration conditions [9]. Our data indicate that β-NADH is also protected in this environment.

The temperature dependence gives information on the ideal temperature to make the measurement. The changes are largely completed by 150 K, and little further change occurs at lower temperature. The results indicate that going to lower temperatures (liquid He, for example) will not increase sensitivity since there is little difference between 77 K (liquid N_2 temperature) and lower temperatures (Figs. 2 and 3).

The difference in the two matrices point to clues as to what type of environment of β-NADH will be contributing most to the β-NADH fluorescence at low temperature. Molecules of β-NADH with water accessibility will have the largest change in fluorescence. This information may be helpful for the interpretation of fluorescence differences in tissues. For instance, it is intriguing why the fluorescence of β-NADH in normal and dysplastic tissue differ under freeze-trapped conditions [18]. It may be that the different composition of the two types of tissue affects the internal water structure. Difference in water in different tissues is supported by many MRI experiments and, indeed, forms a basis for MRI imaging [19]. Differences in water in cells may arise from different composition of macromolecules in different tissues, but may also be due to different compartmentation, since the size of the water pool affects the physical characteristics of water [20].

In conclusion β-NADH fluorescence yield and spectrum are very sensitive to temperature changes. The increase in yield and shift in the emission spectrum should be useful for optimizing conditions for imaging β-NADH location.

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