Conformational Dependence of Intracellular NADH on Metabolic State Revealed by Associated Fluorescence Anisotropy*

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Harshad D. Vishwasrao‡§, Ahmed A. Heikal¶, Karl A. Kasischke, and Watt W. Webb**

From the ‡Howard Hughes Medical Institute, Center for Neurobiology & Behavior, Columbia University, New York, New York 10032, the ¶Department of Bioengineering, Pennsylvania State University, University Park, Pennsylvania 16802, and the §§School of Applied & Engineering Physics, Cornell University, Ithaca, New York 14853

Global analysis of fluorescence and associated anisotropy decays of intrinsic tissue fluorescence offers a sensitive and non-invasive probe of the metabolically critical free/enzyme-bound states of intracellular NADH in neural tissue. Using this technique, we demonstrate that the response of NADH to the metabolic transition from normoxia to hypoxia is more complex than a simple increase in NADH concentration. The concentration of free NADH, and that of an enzyme bound form with a relatively low lifetime, increases preferentially over that of other enzyme-bound NADH species. Concurrently, the intracellular viscosity is reduced, likely due to the osmotic swelling of mitochondria. These conformational and environmental changes effectively decrease the tissue fluorescence average lifetime, causing the usual total fluorescence increase measurements to significantly underestimate the calculated concentration increase. This new discrimination of changes in NADH concentration, conformation, and environment provides the foundation for quantitative functional imaging of neural energy metabolism.

The role of the intrinsic fluorophore NADH as the principal electron donor in glycolytic and oxidative energy metabolism makes it a convenient non-invasive fluorescent probe of metabolic state (1, 2). Traditionally, fluorimetric studies of metabolic dynamics have characterized metabolic states by the total NADH concentration. However, Williams et al. (3) pointed out that the reaction velocity of a given intracellular NADH-linked dehydrogenase depends on the concentration of locally available NADH, i.e. the local concentration of free NADH. Given this thermodynamic importance of free NADH, considerable research has been done to discriminate the intracellular free fraction of NADH.

Analytical chemistry techniques have provided the most accurate and detailed information about intracellular free and total NADH. Pyridine nucleotide extraction (4, 5) gives an absolute measure of the total tissue NAD⁺ and NADH concentrations, while the metabolite indicator method (3, 6–8) has been used to infer both the cytoplasmic and mitochondrial free NAD⁺/NADH ratio from the concentrations of specific cytoplasmic and mitochondrial redox couples. However, these techniques entail destroying the tissue, thereby restricting the study of metabolic dynamics to single shot measurements. Furthermore, these techniques are also intrinsically incapable of resolving spatial variations in the free/bound state ratios.

In contrast, fluorescence spectroscopic techniques (9–15) are non-destructive and readily extendable to an imaging modality to address spatial heterogeneity. These techniques are limited, however, by the ambiguous distinction between free and bound NADH fluorescence. Binding-induced shifts of the emission spectrum (up to ~20 nm) (9–11) are small compared with the width of the NADH spectrum (~150 nm). Fluorescence lifetime is a more sensitive probe of NADH binding because it is enhanced significantly (up to 10 times) (12–14). However, the fluorescence decay of bound NADH is usually multi-exponential with shorter components that can be comparable with the decay time of free NADH (12). This makes it difficult to attribute a fast fluorescence decay component in tissue to free or bound NADH. Since intracellular NADH exists in dynamic pools of both the free form and bound to many different dehydrogenases, the integrated cellular spectra are an uncertain combination of many different spectral influences. What is required is a fluorescence signature that can unambiguously discriminate between free and bound intracellular NADH.

Here we show that global analysis of time resolved fluorescence and anisotropy decays (16, 17) provides simultaneous information on the excited state dynamics and rotational mobility of intracellular NADH. Binding of NADH leads to an exceedingly large (>10 times) increase in the anisotropy decay time (15), reflecting the large difference in the size of the free NADH molecule and the binding enzyme. An anisotropy-based approach therefore offers the most sensitive discrimination of free and enzyme bound NADH. Furthermore, the enhancement in the lifetime of NADH in the intracellular environment provides a measure of the enhancement of its fluorescence quantum yield. By correcting for changes in the fluorescence quantum yield, we can infer fractional changes in the true NADH concentration as a function of metabolic state. We thus present a means of directly monitoring metabolic state dependent changes in the total concentration of NADH and its partition into free and bound pools, thereby bridging the gap between spectroscopy and analytical chemistry.

MATERIALS AND METHODS
Slice Preparation and Perfusion

The protocol for preparing and maintaining transverse hippocampal slices from Sprague-Dawley rats (day 14–20) has been described previously (18, 19). Under these conditions, imaging data revealed that the fluorescence response of slices to hypoxia reached a steady state in ~5
A mixture of 95% O2/5% CO2 was perfused over the hippocampal slice at 2.5 ml/min to maintain the slice in the normoxic state for 10 min prior to acquiring spectra in the normoxic state. The perfusate was then switched to hypoxic ACSF (95% N2/5% CO2) for 10 min prior to acquiring spectra in the hypoxic state. The perfusate containing 95% O2/5% CO2 was equilibrated with the hippocampal slice at a flow rate of 2.5 ml/min to maintain the slice in the normoxic state for 10 min prior to acquiring spectra in the normoxic state. The perfusate was then switched to hypoxic ACSF (95% N2/5% CO2) for 10 min prior to acquiring spectra in the hypoxic state. To ensure reversibility of the metabolic response, spectra were once more acquired after reperfusion with normoxic ACSF for 15 min. The spectra acquired after reperfusion with normoxic ACSF were similar to the spectra acquired before hypoxia, indicating a fully reversible metabolic transition and no significant photodamage during the acquisition of spectra.

**Time-resolved Two-photon Fluorescence Spectroscopy**

Our two-photon time resolved fluorescence setup has been described previously (18, 20). A 670 DCLP dichroic and BGG22 emission filter (350–550 nm/500 nm) were used to separate the NADH fluorescence from the 740 nm two-photon excitation beam. While the intracellular fluorescence consists of both a blue NADH peak and a weak yellow flavin peak (Fig. 1), our choice of emission filter attenuated the contribution of the flavin fluorescence to less than 5%. The blue emission peak also receives a contribution from intracellular NADPH that is spectrally identical to NADH. However, the low concentration of NADPH in the hippocampus and its minimal involvement in energy metabolism makes this fluorescence a minor and roughly constant background signal (see “Discussion”).

The acquisition of both time resolved fluorescence spectra and anisotropy decays using time correlated single photon counting on our apparatus has been described in detail elsewhere (20), so we shall be brief here. To maximize fluorescence collection efficiency during tissue measurements, the excitation beam was maintained stationary with respect to the optical axis of the microscope, and the sample was scanned using a computer controlled x-y stage (SCAN IM, Merzhäuser) so as to effectively raster scan the excitation beam in a 100 × 100 μm square in the stratum radiatum. This scanning stage technique reduces photodamage by distributing the exposure over an area ∼10 times the area of the focused excitation beam.

Taking advantage of the optical sectioning ability of two-photon excitation, fluorescence was excited at 70–100 μm within the slice. This depth was chosen to avoid tissue near the surface of the brain slice that might be damaged during preparation.

**Definition of Fluorescence and Anisotropy Decay**

The polarization of the fluorescence emitted by a sample consists of parallel terms $I_{\parallel}$ and perpendicular component $I_{\perp}$. These orthogonal directions are defined with respect to the polarization of the excitation light. In the experiments described throughout this work, these two fluorescence components are typically measured sequentially, rotating an analyzer before the photodetector and integrating the fluorescence signal for equal times. Having measured these two fluorescence decay curves, we can calculate the total fluorescence decay curve $I(t)$ as the magic angle fluorescence (Eq. 1) also known as the magic angle fluorescence decay and the rotational anisotropy decay curve $r(t)$, defined by Equations 1 and 2, respectively.

$$I(t) = I_{\parallel}(t) + 2G I_{\perp}(t) \quad (Eq. 1)$$

$$r(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)} \quad (Eq. 2)$$

Here, the G-factor is the degree to which the optical detection path preferentially transmits one polarization over the other. The total fluorescence decay is a measure of the population of the excited state. The anisotropy of the fluorescence is the degree to which molecular orientation polarizes the emitted fluorescence. The anisotropy is initially non-zero because the excitation beam polarization selectively excites a particular orientation of molecules. The decay of the anisotropy therefore measures the decay of net molecular orientation due to diffusive motion. The characteristic decay time, known as the rotational diffusion time, characterizes the timescale of this diffusive motion.

**Functional Form of Fluorescence Decay**

The total fluorescence decay was calculated from the recorded parallel and perpendicular fluorescence decays according to Equation 1. The total fluorescence decay $I(t)$ was then fit using either the commercial Becker & Hickl SPCImage 2.4 fitting program or custom-written fitting routines in Matlab. These programs utilize non-linear least squares fitting algorithms to fit the fluorescence decay with the following functional form.

$$I(t) = \left( I_{\text{baseline}} + I_0 (a_0 \delta t - t) + \sum_{i=1}^{n} a_i \exp(-(t - t_i)/\tau_i) \right) \times R(t) \quad (Eq. 3)$$

**Definition of Terms in Fitting Equations**

**Decay Kinetics**—The sum of exponential terms in this expression represents the excited state fluorescence dynamics of the molecule. Each individual exponential term $a_i \exp(-t/\tau_i)$ represents the decay of the excited state via a particular pathway. These different pathways can be associated with different fluorophores, different conformations of a fluorophore, or simply different excited states to ground state transitions in a fluorophore. For the purpose of general discussion, we will simply refer to the physical sources of these exponential decay components as species, since a more precise physical identification is case specific. The amplitude $a_0$ of an individual decay component is equal to the fractional population of the species associated with it. Hence it follows that the sum of the fractional populations must be unity (see Equation 4).

$$\sum_{i=1}^{n} a_i = 1 \quad (Eq. 4)$$

The lifetime of a decay component is equal to the inverse of the total transition rate from the excited state to the ground state, i.e. Equation 5.

$$\tau = \frac{1}{k_f} = \frac{1}{k_r + k_{wr}} \quad (Eq. 5)$$

where $k_r$ is the radiative rate (the rate of relaxation from the excited to the ground state involving the emission of a fluorescence photon) and $k_{wr}$ is the total non-radiative rate (the net rate of transition to the ground state not involving the emission of a fluorescence photon). The fluorescence quantum yield is by definition the fraction of those transitions back to the ground state which involve emitting a fluorescence photon.

**Scattered Excitation Light**—The δ function term $a_0 \delta t - t_0$ in Equation 3 represents any minor backscattered excitation light that bleeds through the emission filter. Since the excitation pulse is ~100 fs in duration, the backscattered light will also be comparable in duration, with some temporal stretching due to the variability in pathlength of backscattered photons reaching the detector. The duration of this backscattered pulse is far below the temporal resolution of our detector (~10 ps), and hence we can approximate it with a δ function. While this term can be excluded for measurements in solution, it is necessary in highly scattering tissue such as brain slices.
Amplitudes and Offsets—Equation 3 in the baseline or background fluorescence, approximated as constant for all times. \( \lambda_p \) is the peak fluorescence at time \( t_p \), where \( t_p \) is the temporal offset of the fluorescence decay from the start of the acquisition window.

Response Function—\( R(t) \) is the measured response function of the system, recorded using second harmonic generation from powder crystals of KDP* or rat tail collagen. This response function is convolved,

\[
f(t) \otimes R(t) = \int_{-\infty}^{\infty} R(t')f(t - t')dt'
\]

(Eq. 7)

with the guess fit function and then compared with the observed decay.

Derived Quantities

Once the total intensity decay fit parameters have been obtained by the fitting program, we can calculate a number of useful derived quantities:

1) the average lifetime (\( \langle \tau \rangle \)) of the fluorescence;

\[
\langle \tau \rangle = \sum_{i=1}^{n} a_i \tau_i
\]

(Eq. 8)

2) the fraction of the total fluorescence generated by each species \( i \) is \( f_i \);

\[
f_i = \frac{a_i \tau_i}{\sum_{i=1}^{n} a_i \tau_i}
\]

(Eq. 9)

3) the change in the concentration of species \( i \) upon hypoxia.

\[
\frac{C_{i, \text{hypoxia}}}{C_{i, \text{normoxia}}} = \frac{a_{i, \text{hypoxia}}}{a_{i, \text{normoxia}}} = \frac{I_{i, \text{hypoxia}}}{I_{i, \text{normoxia}}} = \frac{C_{i, \text{hypoxia}} - C_{i, \text{normoxia}}}{C_{i, \text{normoxia}}} + 1
\]

(Eq. 10)

Functional Form of Fluorescence Anisotropy Decay

The fluorescence anisotropy decay of a freely rotating molecule in an isotropic environment is usually described adequately by a single exponential,

\[
r(t) = r_0 \exp(-t/\theta)
\]

(Eq. 11)

where \( \theta \) is the rotational diffusion time of the molecule and \( r_0 \) is the initial anisotropy. The anisotropy decays of many conventional fluorophores, e.g. fluorescein, coumarin, as well as NADH in solution or NADH fully bound to malate dehydrogenase in solution, were found to follow this functional form. The initial anisotropy \( (r_0) \) is solely a property of the fluorophore itself and is related to the angle between the absorption and emission dipoles \( (\delta) \) by Equation 12.

\[
r_0 = \frac{2a}{2a + 3 \left(3\cos^2 \delta - 1 \right) / 2}
\]

(Eq. 12)

Based on the number of photons absorbed \( (a) \), the maximum theoretical \( r_0 \) values are 0.4 and 0.57 for one-photon and two-photon events, respectively (corresponding to collinear dipoles, without depolarizing processes, i.e. \( \delta = 0 \)). The higher theoretical maximum of two-photon fluorescence anisotropy (compared with one-photon) offers a greater dynamic range, and therefore sensitivity, for the measurement of rotational anisotropy.

The rotational diffusion time, on the other hand, depends not only upon the fluorophore molecule itself but also upon the environmental conditions. This dependence may be summarized by the Stokes-Einstein equation (Equation 13).

\[
\theta = \frac{1}{6D_{\text{rot}}} \times \frac{\eta V}{kT}
\]

(Eq. 13)

where \( D_{\text{rot}} \) is the rotational diffusion coefficient, \( V \) is the hydrodynamic volume of the molecule, \( \eta \) is the local viscosity of the environment, \( T \) is the absolute temperature, and \( k \) is the Boltzmann constant. This relation approximates the hydrodynamic volume (molecule + hydrodynamic shell) of the molecule to be spherical, which is a reasonable approximation for NADH (21). We use this relation later to differentiate between free and bound NADH as well as to estimate the viscosity of the intracellular environment.

While a single exponential anisotropy decay approximates the decay of a free fluorophore, more complicated decays are also possible under certain circumstances. Of particular relevance here, associated anisotropy is the form of fluorescence anisotropy arising from a mixture of fluorophore species, each fluorophore species having its own distinct fluorescence and fluorescence anisotropy decays. The fluorescence anisotropy decay from the mixture is the average of the fluorescence anisotropy decays of the individual species, weighted by their respective fluorescence decays. Fitting the anisotropy decay with the associated anisotropy model associates a distinct rotational diffusion time with each lifetime decay component as described by Equation 14 (22, 23).

\[
r(t) = \frac{\sum_{i=1}^{n} a_i \exp(-t/\tau_i) r_0 \exp(-t/\theta)}{\sum_{i=1}^{n} a_i \exp(-t/\tau_i)}
\]

(Eq. 14)

where each species \( i \) has its own set of total intensity decay parameters \( (a_i, \tau_i) \) and set of anisotropy decay parameters \( (r_0, \theta) \).

Fitting Protocol

The analysis of anisotropy decay curves is somewhat more difficult than a simple non-linear least squares fit provided by most commercial data analysis software. Hence, it was necessary to write custom programs to perform these fits. The fitting protocol employed during this analysis is a form of global analysis specifically modified for analyzing associated anisotropy decays.

Recall that the actual measured fluorescence decays are the parallel \( (I_{||}(t)) \) and perpendicular \( (I_{\perp}(t)) \) polarization components of the fluorescence decay. From these, one can calculate the anisotropy decay curve using Equation 15.

\[
r(t) = \frac{I_{||}(t) - GI_{||}(t)}{I_{||}(t) + 2GI_{||}(t)} = \frac{\sum_{i=1}^{n} a_i \exp(-t/\tau_i) r_0 \exp(-t/\theta)}{\sum_{i=1}^{n} a_i \exp(-t/\tau_i)}
\]

(Eq. 15)

In practice, however, we do not actually calculate \( r(t) \), but rather we calculate the numerator and denominator separately. By convention, the numerator is called the difference curve \( (D(t)) \) and the denominator is called the sum curve \( (S(t)) \), simply reflecting the operation between the parallel and perpendicular fluorescence components. Both the sum and difference curves are sums of exponentials. Furthermore, in practice, the functional form of each is more complicated than a simple sum of exponential terms. The most general form of both the sum and difference curves is analogous to Equation 3; that is, there are terms to account for baseline, scattered light, temporal offset, and detector response.

\[
S(t) = I_{||}(t) + 2GI_{||}(t) = I_{\text{baseline,}S} + I_{\text{dipole}}(a_{0,0}\theta(t - t_0) + \sum_{i=1}^{n} a_i \exp(-(t - t_i)/\tau_i)) \otimes R(t)
\]

(Eq. 16)

\[
D(t) = I_{||}(t) - GI_{||}(t) = I_{\text{baseline,}D} + I_{\text{dipole}}(a_{0,0}\theta(t - t_0) + \sum_{i=1}^{n} a_i \exp(-(t - t_i)/\tau_i)) \otimes R(t)
\]

(Eq. 17)

To fit these individually with a minimization algorithm, we must define a reduced chi-squared for both these curves,

\[
\chi^2 = \frac{1}{n - p_{s} - p_{d}} \sum_{t=1}^{n} \left( S(t_i) - S(t_i) \right)^2
\]

(Eq. 18)
where $n$ is the number of data points in the curve, $p_G$ is the number of fitting parameters, and $S_{in}$ is the trial fitting function. An analogous definition can be made for the difference curve $D(t)$ but with a slightly different weighting factor.

\[
D(t) = I(t) - G I(t)
\]

\[
\chi^2_{global} = \frac{1}{2n - (p_G + p_D - p_{global})}
\]

where $p_{global}$ is the number of global parameters. The fitting protocol is schematically represented in Fig. 2. Thus, by performing a global fit, we can simultaneously fit $S(t)$ and $D(t)$ and automatically satisfy the constraint that certain shared parameters must have the same values in both fits. The global fit then provides us with both the total intensity decay parameters ($a_i, \tau_i$) and the anisotropy decay parameters ($r_{ai}, \theta_i$) in a single minimization operation with an improved resolution on the shared parameters.

This global fitting protocol was tested by using a standard sample of fluorescein. Fluorescein was found to have mono-exponential fluorescence and anisotropy decays with a lifetime of 3.9 ns, a rotational diffusion time of 136 ps and a two-photon initial anisotropy of 0.39. These values are in good agreement with values previously reported in literature (24, 25).

**RESULTS**

**Tissue Fluorescence Measurement—** Time-resolved fluorescence decays were recorded from the stratum radiatum of the CA1 layer of rat hippocampal slices. This layer consists largely of axo-dendritic network with scattered astrocytes (Fig. 3A). Each slice was subjected to a 10-min hypoxic episode during which the intrinsic fluorescence increased in intensity (Fig. 3B) by an average of 48 ± 26%. Reperfusion with oxygenated solution returned the integrated fluorescence to approximately baseline levels (Fig. 3C), with a slight hyperoxidation due to post-hypoxic elevated mitochondrial metabolism (26, 27). This reversible response indicates negligible photodamage. The intrinsic fluorescence from the hippocampal slice consists of both a blue NADH peak and a yellow flavin peak (Fig. 1) that respond differently to hypoxia. However, our choice of emission filter ensured that the contribution from the NADH peak to the detected fluorescence was greater than 95%.

Fluorescence decays at parallel and perpendicular polarizations were measured sequentially and used to calculate the total (magic angle) fluorescence decay and the anisotropy decay. Each total fluorescence and anisotropy decay pair was fit simultaneously using a global analysis to obtain the parameters characterizing both the excited state lifetime and the rotational mobility.

The average of the total fluorescence decays ($n = 6$) under both normoxic and hypoxic conditions are shown in Fig. 4A. The tissue fluorescence is found to have four exponential decay components (we will call these species 1 through 4) whose lifetimes ($\tau_i$) and amplitudes, or relative concentrations ($a_i$), are summarized in Table I. The lifetimes ($\tau_i$) are characteristic decay times of the excited state of each species. The amplitudes ($a_i$) are the relative concentrations of the species. In response to hypoxia, there is a modest decrease in all but the longest time constant. The amplitudes, however, are heterogeneous in their response. There are decreases in the amplitudes of the two longer lifetime species in favor of the two shorter lifetime species. The net effect of these changes is a decrease in the average lifetime from 948 ± 46 ps (normoxia) to 780 ± 17 ps (hypoxia). From the total fluorescence decay parameters, we can calculate a number of physically important quantities. First, the changes in the absolute concentrations of the individual species ($\Delta C_i$) are calculated to be: 86 ± 45% (species 1), 104 ± 43% (species 2), 39 ± 25% (species 3), and 58 ± 35% (species 4). The increase in the total concentration of NADH upon hypoxia was calculated to be 81 ± 39%, which is substantially greater than the change in the total fluorescence (48 ± 26%) due to the decrease in the fluorescence average lifetime.

The large errors are due to significant variation in the integrated fluorescence response to hypoxia and not to large errors in the fitting parameters. Second, the fractional contributions to the fluorescence ($f_i$) from each species during normoxia can be calculated to be: 7% (species 1), 20% (species 2), 46% (species 3), and 27% (species 4). Note that while species 1 has the largest concentration (44%), it contributes the least to the total fluorescence due to its short lifetime.

The total fluorescence decay exhibits a spike at $t_r$ resembling the response function due to backscattered excitation light from the tissue that increased by 47 ± 20% upon hypoxia. This increase in scattered light has been observed during sustained hypoxia, and although its cause is not fully understood, it is possibly due to mitochondrial swelling (28–30).

To further characterize the fluorescent species, we calculated...
The average fit parameters are summarized in Table II. Anisotropy decays exhibit the functional form of associated anisotropy model in our fitting protocol, we determined the rotational anisotropy decays from the intrinsic tissue parallel and perpendicular fluorescence components. Typical anisotropy decays (Fig. 4) exhibit a rapid initial decay, followed by a rise and then a second slower decay. This form is characteristic of fluorescence anisotropy arising from a mixture of fluorophore species with each species having its own distinct fluorescence decay and fluorescence anisotropy decay and is known as associated anisotropy (22, 23). By utilizing the associated anisotropy model in our fitting protocol, we determined the rotational diffusion time and initial anisotropy of each detected species of the total fluorescence decay and thereby characterized the conformations of NADH as a function of metabolic state.

The anisotropy parameters \( (r_{0i}, \theta_i) \) obtained by the fit are summarized in Table II. Of particular note, the shortest lifetime species (species 1) has a fast rotational diffusion time of 318 ± 101 ps during normoxia that decreases upon hypoxia to 230 ± 58 ps. In contrast, all three longer lifetime species are essentially immobile, with large rotational diffusion times that are insensitive to the tissue metabolic state. The two-photon initial anisotropies of all the species are also insensitive to metabolic state, except species 2, which exhibits a significant decrease of the initial anisotropy from 0.33 ± 0.01 to 0.25 ± 0.02, indicating an increased mode of depolarization faster than our time resolution.

**Comparative Measurement of NADH in Solution**—The photophysical properties of the intracellular fluorescence species can be used to physically identify them by comparatively considering the photophysical characteristics of NADH in solution. Free aqueous NADH at room temperature exists in two conformations: folded (stacked nicotinamide and adenine rings) and extended (no such stacking). We measure the total fluorescence decay to be bi-exponential with lifetimes (and amplitudes) 350 ps (0.77) and 760 ps (0.23) giving an average lifetime of 444 ps. The two lifetime components are comparable with those reported in literature and are believed to arise from the extended and folded populations respectively (12, 21). The fluorescence anisotropy decay was found to be mono-exponential, with a rotational diffusion time of 290 ps and an initial anisotropy of 0.38 (Fig. 5, blue curve). The rotational dynamics reported here are consistent with those reported by Couprie et al. (21) who further showed that the NADH rotational diffusion time exhibits a Stokes-Einstein dependence on viscosity and temperature (Equation 13). Based on the Stokes-Einstein dependence measured by Couprie et al. (21), we expect a reduced rotational diffusion time for free aqueous NADH of −150 ps at the temperature of tissue in our experiments (−32 °C).

NADH fluorescence and anisotropy are sensitive to enzyme binding. As an example, we titrate the large NADH-binding Krebs cycle enzyme mitochondrial malate dehydrogenase (mMDH, molecular mass = 70 kDa) against NADH (molecular mass = 665 Da). At a sufficiently high concentration of mMDH ([NADH binding sites]/[NADH] = 4), all available NADH is bound. In this case, the fluorescence decay is bi-exponential with components 602 ps (0.72) and 1.33 ns (0.28) yielding an average lifetime of 806 ps. The anisotropy decay as a mono-exponential with an initial anisotropy of 0.49 and a rotational diffusion time of 30 ns (Fig. 5, red curve). The long, single exponential anisotropy decay implies that the NADH fluoro-
Intracellular Free NADH Revealed by Fluorescence Anisotropy

Table I
Summary of the fluorescence lifetime parameters

| Species | $a_1$ (ps) | $\tau_1$ (ps) | $a_2$ (ps) | $\tau_2$ (ps) | $a_3$ (ps) | $\tau_3$ (ps) | $a_4$ (ps) | $\tau_4$ (ps) |
|---------|-----------|--------------|-----------|--------------|-----------|--------------|-----------|--------------|
| Normoxia| 0.44 (0.02) | 155 (25) | 0.32 (0.01) | 599 (49) | 0.20 (0.01) | 2154 (79) | 0.042 (0.003) | 6040 (13) |
| Hypoxia | 0.45 (0.02) | 129 (7) | 0.36 (0.01) | 530 (17) | 0.16 (0.01) | 1975 (72) | 0.0037 (0.001) | 6054 (22) |

% change upon hypoxia | +2 (6) | -15 (15) | +13 (6) | -11 (9) | -22 (7) | -8 (6) | -12 (5) | +2 (0.5) | -18 (4) |

Table II
Summary of rotational anisotropy parameters

| r_{c1} | r_{c2} | r_{c3} | r_{c4} | r_{c5} | r_{c6} | r_{c7} | r_{c8} |
|--------|--------|--------|--------|--------|--------|--------|--------|
| Normoxia | 0.42 (0.04) | 318 (101) | 0.33 (0.01) | 8443 (2495) | 0.41 (0.02) | $\infty$ | 0.36 (0.02) | $\infty$ |
| Hypoxia | 0.43 (0.06) | 230 (58) | 0.25 (0.02) | 8070 (905) | 0.39 (0.01) | $\infty$ | 0.33 (0.02) | $\infty$ |

Table III
Summary of the identification of the observed fluorescence species based upon a combined consideration of their excited state and rotational dynamics

The relative concentrations are given in arbitrary units such that the sum of the concentrations during normoxia is 100.

| Species | Identification | Concentration | Increase upon hypoxia |
|---------|----------------|--------------|----------------------|
|         |                | Normoxia     | Hypoxia              | %                     |
| 1       | Free           | 44           | 80                   | 82                   |
| 2       | Bound          | 32           | 63                   | 97                   |
| 3       | Bound          | 20           | 27                   | 35                   |
| 4       | Bound          | 4            | 6                    | 50                   |

Anisotropy decays from solutions of free NADH (blue), NADH fully bound to mMDH (red), and a mixture of free and bound NADH (partially bound) (green). Fully free and fully bound NADH show simple exponential decays with grossly different rotational diffusion times that reflect the differing sizes of NADH and mMDH. The anisotropy decay of the mixture of free and bound NADH exhibits associated anisotropy, similar in appearance to the decays observed in tissue.

The fluorescence lifetime parameters are given as mean (standard deviation) for $n = 6$.

This high rotational mobility is consistent with this species being free NADH in a slightly viscous environment. The lifetime of species 1 is comparable with the lifetime of free extended NADH but substantially shorter than the lifetime of free folded NADH. Taken together, the lifetime and anisotropy parameters for species 1 suggest that it is free extended NADH in a slightly viscous environment. Species 2 has a lifetime that is consistent with being either folded NADH or enzyme-bound NADH. Its rotational diffusion time (~8 ns), however, is almost 2 orders of magnitude greater than that of free NADH, indicating that this species is also bound NADH. This species undergoes a significant decrease in its initial anisotropy (0.33–0.25) upon hypoxia, indicating an enhanced mode of fast depolarization, e.g. a fast segmental motion of the nicotinamide ring within an enzyme, or a specific interaction in the binding sites which increases the angle between the nicotinamide absorption and emission dipoles. While the cause is uncertain, we can still identify this species as enzyme bound on the basis of its diffusion time. The two longest lifetime species (species 3 and 4) are rotationally immobile on the timescale of our measurement window (~10 ns), and their lifetimes are much greater than the lifetimes of either folded or extended aqueous NADH. Both the highly enhanced lifetime and rotational immobility suggest that these two species are NADH bound to enzymes. The identification of species 2–4 as enzyme bound does not imply that there are only three different enzymes binding NADH. In reality, there are known to be many different enzymes binding NADH, and their net fluorescence is observed as components 2–4 (3, 9, 11, 12, 31).

It is noteworthy that we do not observe the expected ~600–700-ps fluorescence decay component of free folded NADH in brain slices. However, it is difficult to say whether this reflects an inability to separately resolve its decay or a genuinely small intracellular concentration of free folded NADH.

From the identification of these species, the relative concentration of free NADH is given by the amplitude of species 1 ($a_{free} = a_1$), while the relative total concentration of bound...
NADH is given by the sum of the amplitudes of the three bound species \( \sigma_{\text{bound}} = \sigma_1 + \sigma_2 + \sigma_3 \). The ratio of the free/bound NADH \( \frac{\sigma_{\text{free}}}{\sigma_{\text{bound}}} \) can then be calculated to be 0.78 ± 0.05 during normoxia and remains essentially unchanged upon hypoxia. Even though hypoxia does not affect the ratio of free NADH to the total concentration of bound NADH, it does affect the relative populations of the three enzyme bound species (Table I), indicating a redistribution of NADH among different enzymes. Hence while the free NADH concentration appears to remain approximately equal to the total concentration of bound NADH, its distribution among various bound species changes with its chemical potential, indicating a range of binding constants and cofactor concentrations in the intracellular environment.

Calculating the Local Intracellular Viscosity—Having identified species 1 as free NADH, we can use its rotational diffusion time with the Stokes-Einstein relation (Equation 13) for free NADH measured by Couprie et al. (21) to calculate the local intracellular viscosity. In this way, the local viscosity experienced by free intracellular NADH is calculated to be 1.6 ± 0.5 mPa·s (2.1 ± 0.7 relative to water at 32 °C) during normoxia and decreases upon hypoxia to 1.1 ± 0.3 mPa·s (1.5 ± 0.4 relative to water at 32 °C).

**DISCUSSION**

A number of intrinsic fluorophores are present in hippocampal tissue; however only NADH, NADPH, and flavins are of sufficient concentration to yield a detectable fluorescence signal under two-photon excitation at 740 nm. While the flavin signal can be spectrally separated (see Fig. 1), there is no known photophysical means of discriminating NADH and NADPH. It is therefore necessary to consider the relative contributions of these two reduced pyridine nucleotides to the measured intracellular fluorescence.

The concentrations of pyridine nucleotides have been measured by high performance liquid chromatography in mouse hippocampus (4). It was found that the total concentration of diphosphopyridine nucleotides (NADH and NADPH) is greater than that of triphosphopyridine nucleotides (NADPH and NADP⁺) by a factor of 10. The concentration of the reduced fluorescent species NADH was found to be ~5 times greater than that of the fluorescent NADPH. Furthermore, the enhancement of mitochondrial NADH quantum yield due to environmental effects has been estimated to be a factor of 1.25–2.5 greater than that of NADPH (33). This implies that the contribution of NADPH to the intrinsic fluorescence, while not insignificant, represents a small fraction of the NADH contribution.

Like NADH, the ratio of the fluorescent NADH to its non-fluorescent oxidized form NADP⁺ is dependent upon the relative rates of NADH source/sink reactions. However, NADH and NADPH are distinct in their biochemical roles: NADH being largely restricted to energy metabolism and NADPH largely to reductive biosynthesis (34). It has been reported that the chemical kinetics of NADH and NADPH differ in certain reactions (35). However, tissue analyses of pyridine nucleotide content in heart and liver have confirmed that the fluorescence changes during the normoxic-anoxic transition are dominated by changes in NADH with only a minor (~10%) contribution from NADPH (36). Hence, NADPH represents a minor fluorescence background that is roughly constant with respect to metabolic perturbations.

With the caveat of a small, metabolically inert signal from NADPH, intrinsic fluorescence can be used to characterize the conformation and environment of intracellular NADH. Enzyme binding of NADH has been characterized by a number of fluorescence techniques that are differently sensitive to binding. The weakest effect is the shift of the NADH emission peak upon binding, which is typically less than 10% (\( \Delta \lambda/\lambda < 0.1 \)). The lifetime of NADH is more sensitive to enzyme binding and can be enhanced by a factor of \( \Delta t/\tau \sim 1-10 \). Fluorescence anisotropy offers an even greater sensitivity to enzyme binding due to the large difference in size between NADH (molecular mass = 665 Da) and the enzymes that tightly bind it, e.g. mitochondrial malate dehydrogenase has a molecular mass of 70 kDa. Enzyme binding increases the rotational diffusion time of NADH by more than an order of magnitude (\( \Delta \theta/\theta \sim 10-100 \)), rendering the fluorophore effectively immobile on our time scale of measurement. Hence, measuring the rotationally mobile versus immobile intracellular NADH offers the most sensitive fluorescence probe of the free versus bound conformation.

Based on our measurements of rotational anisotropy, we conclude that intracellular NADH exists in both free and bound forms in comparable concentrations (free/bound = 0.78). However, it is the bound NADH that dominates (93%) the total tissue fluorescence due to its substantially enhanced lifetime. This dominance of the bound NADH fluorescence has led analyses of the wavelength resolved tissue fluorescence spectra to prematurely conclude that tissue NADH is almost completely enzyme bound (9). In contrast, our results indicate that free NADH is in fact significant in concentration if not in fluorescence.

The ratio of free NADH to individual enzyme bound species of NADH is found to change significantly upon hypoxia, while the ratio of free NADH to total bound NADH does not. This indicates a redistribution of NADH among enzyme binding sites. Specifically, there is a large increase in the concentration of free NADH and the concentration of NADH bound to enzymes that cause low lifetime enhancement. The concentration of NADH bound to those enzymes causing large lifetime enhancement increases to a lesser extent. The net effect of this redistribution of NADH among binding sites is a decrease in the average lifetime of the intrinsic fluorescence, causing the observed fluorescence increase (48%) to underestimate the calculated concentration increase (81%). Since the ratio of free to bound NADH does not change upon hypoxia, this calculated concentration increase applies to both free and bound pools of NADH.

In comparison, assays of chemically extracted NADH from cerebral cortical brain slices showed that the total NADH concentration increased by 107% upon acute hypoxia (5). Metabolite indicator methods reveal a 105% increase in the free mitochondrial NADH (8) in brain upon hypoxia. These concentration increases are clearly much closer to our calculated concentration increase than to our observed fluorescence increase. However, a strict comparison should not be drawn between our results and the results of the metabolic indicator method because NADH increases are dependent upon the degree of hypoxia as well as the region of the brain, both of which may differ from our experiments.

In relating fluorescence changes to concentration changes, we have assumed that changes in the quantum yield are proportional to changes in the lifetime. This is only strictly true if the radiative rate remains constant (Equation 6). The radiative rate depends on the overlap of excited and ground states, which can be expressed as a function of the fluorescence and absorption spectral line shapes (shape of the normalized spectra) as expressed by the Strickler-Berg relation (37). We find that the line shape of the NADH portion of the intrinsic fluorescence changes only slightly upon hypoxia (Fig. 1), suggesting that the average radiative rate of intracellular NADH is not significantly altered. However, this is not conclusive as we do not know the behavior of the absorption spectral line shape.
We have further assumed here that the local refractive index of NADH does not change during enzyme binding in an aqueous buffer. However, the environment within several wavelengths of an enzyme bound fluorophore, consisting of the binding site of the enzyme as well as the solution surrounding the enzyme itself, can affect the radiative rate of the fluorophore (38). As such, the variation of the refractive index on a microscopic scale in tissue remains an uncertainty in this research.

The rotational dynamics of intracellular free NADH indicate a relatively low viscosity (~1.6 mPa s). This is not unexpected because fast rotational diffusion and conformational changes take place on length scales that are short and therefore sensitive to only the immediate fluid phase viscosity and molecular crowding (together known as the effective viscosity) (39). Local fluid phase viscosities comparable with that of water have been observed in a number of cells and subcellular compartments, including mitochondria (39–42).

The decrease in the intracellular viscosity upon hypoxia (to 1.1 mPa s) possibly reflects a dilution of the intracellular environment caused by the osmotic swelling of the cell soma and mitochondria. Sustained hypoxia is known to induce osmotic cell swelling and an increase in scattered light (28–30). This increase in scattered light is believed to be due to mitochondrial swelling (28) in response to hypoxia induced spreading depression. It is thought that mitochondrial swelling increases the cross-section of scatterers and therefore the scattering signal, assuming that there is a significant difference between the refractive index of mitochondria and of the cytosol. Given that we also observe an increase in scattered light upon hypoxia, it is plausible that compartmental swelling and the resulting dilution is reducing the intracellular viscosity.

Our results have demonstrated that the response of NADH to hypoxic metabolic inhibition is more complicated than a simple concentration increase. Changes occur in not only the concentration but also in the conformation and the environment of intracellular NADH. We have presented here a means to discriminate and monitor these effects, enabling a more precise measurement of the dynamics of the actual intracellular NADH concentration and its partition into free and bound pools. It is a logical next step to apply this time resolved fluorescence-anisotropy probe to an imaging modality to explore the spatial heterogeneity of the NADH concentration and conformation, both on an intercellular (e.g. neurons versus astrocytes) and also on a subcellular compartment level.

There has been a resurgence in the use of intrinsic NADH fluorescence as a probe of a wide range of interesting cellular and tissue phenomena, ranging from neuro-metabolic coupling (18) to nuclear transcription regulation (43) to enzyme kinetics within intact cells (44). The fluorescence based assay of NADH concentration and conformation presented here enhances our ability to quantify precisely the role of NADH in these phenomena and to develop an accurate picture of NADH biochemistry and cellular energy metabolism in vivo.

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Harshad D. Vishwasrao, Ahmed A. Heikal, Karl A. Kasischke and Watt W. Webb

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