

Fluorescence quenching of thionine by reduced nicotinamide adenine dinucleotide

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Abstract—Reduced nicotinamide adenine dinucleotide (NADH) is shown to quench the fluorescence of thionine. Quenching of thionine is extremely efficient with a half quenching concentration of only 16.1×10^{-6} M NADH. A Stern–Volmer plot is linear over the NADH concentration range from 1 to $20 \mu\text{M}$. The corresponding Stern–Volmer quenching constant is $6.2 \times 10^4 \text{ M}^{-1}$ and the limit of detection for NADH measurements is 1.6×10^{-6} M. Process of quenching is attributed to the formation of an exciplex between thionine and NADH. Potential analytical features of this system are discussed.

INTRODUCTION

MANY bioanalytical procedures have been developed based on the measurement of reduced nicotinamide adenine dinucleotide (NADH). The absorbance [1], fluorescence [1, 2], and electrochemical properties [3, 4] of this molecule have been used extensively in this regard. Assays have also been developed based on the detection of NADH with either a chemiluminescent [5–6] or bioluminescent [7] reaction scheme.

In continuation to our work on molecular fluorescence [8–11], in this paper we report on an extremely efficient quenching of thionine by NADH. The significance of this discovery is the potential to develop a new assay procedure for NADH based on fluorescence quenching. Owing to the vast amount of bioanalytical chemistry that relies on the detection of this important compound, a unique assay for NADH by a fluorescence quenching process could have tremendous impact on the practice of analytical chemistry in clinical and biomedical laboratories.

EXPERIMENTAL

Thionine (3,7-diaminophenothiazin-5-ium) was purchased from Aldrich Chemical Co. (Milwaukee, WI). NADH was purchased from Sigma Chemical Co. (St. Louis, MO). Both compounds were used as obtained from the supplier. All the test solutions were prepared immediately before use with Type I, reagent grade water prepared with a Milli-Q 3 house water purification unit (Millipore Corp., Bedford, MA).

Stock solutions of NADH and thionine were prepared by dissolving appropriate amounts of these materials in a suitable quantity of a 0.1 M, pH 7.0 phosphate buffer. The concentrations for these stock solutions were 1.0 and 0.5 mM for NADH and thionine, respectively. Final test solutions were prepared by mixing the required amounts of these two stock solutions and diluting to volume with buffer.

All fluorescence measurements were made with an Aminco SPF-500C spectrophotometer from SLM Instruments (Urbana, IL). This instrument was equipped with a 300 W xenon arc lamp (LX 300150) as the source and a Hamamatsu R928P photomultiplier tube as the detector. Absorption spectra were recorded using a Perkin–Elmer $\lambda 5$ spectrophotometer.

Fluorescence quenching measurements were made by recording a series of standard solutions in which the concentration of NADH ranged from 0 to $20 \mu\text{M}$. The concentration of thionine was

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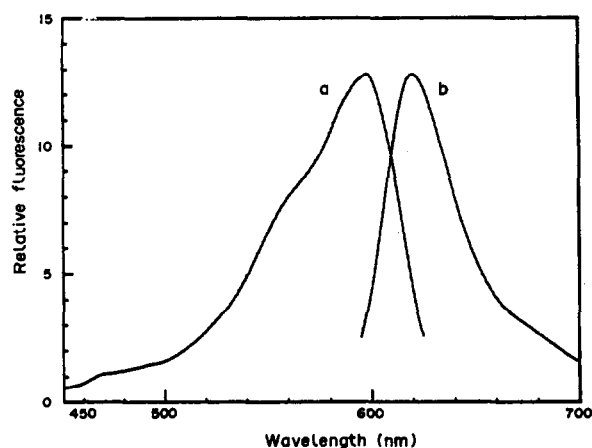


Fig. 1. Excitation and fluorescence spectra of thionine in a 0.1 M, pH 7.0 phosphate buffer.

kept constant at $1 \mu\text{M}$ to avoid self-quenching or inner-filter effects. The excitation wavelength was 596 nm for these spectra. A 2 nm bandpass was used throughout for both the excitation and emission monochromators. All measurements were made in a standard quartz sample cell ($1 \times 1 \text{ cm}$) at room temperature ($22 \pm 1^\circ\text{C}$).

RESULTS AND DISCUSSION

The fluorescence of thionine is characterized by broad excitation (*ca* 400–640 nm with a peak at 596 nm and a shoulder at 560 nm) and emission spectra (*ca* 580–700 nm with a peak at 620 nm). Figure 1 shows these spectra for a $1 \mu\text{M}$ solution of thionine in a phosphate buffer at pH 7.0, and are in agreement with the literature [12].

The ability of NADH to quench the fluorescence of thionine is demonstrated by the series of thionine emission spectra plotted in Fig. 2. These spectra reveal that the fluorescence intensity of thionine decreases as the concentration of NADH increases. The data in this figure also show that the position of the thionine emission band is independent of the NADH concentration.

The fluorescence quenching data have been analyzed according to the Stern–Volmer relationship:

$$I_0/I = 1 + K_{\text{SV}}[Q]$$

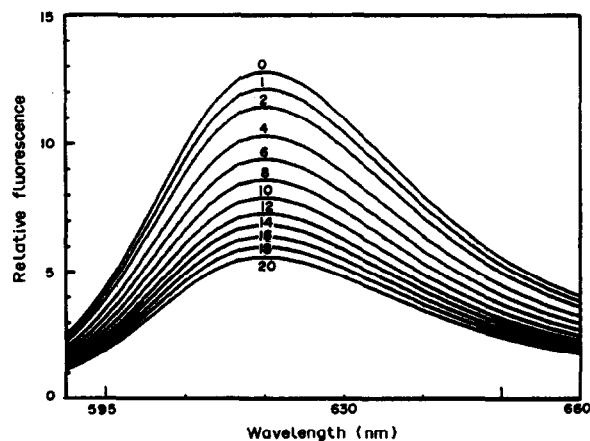


Fig. 2. Effect of NADH concentration on the emission spectra of thionine. Values specify the micromolar concentration of NADH used.

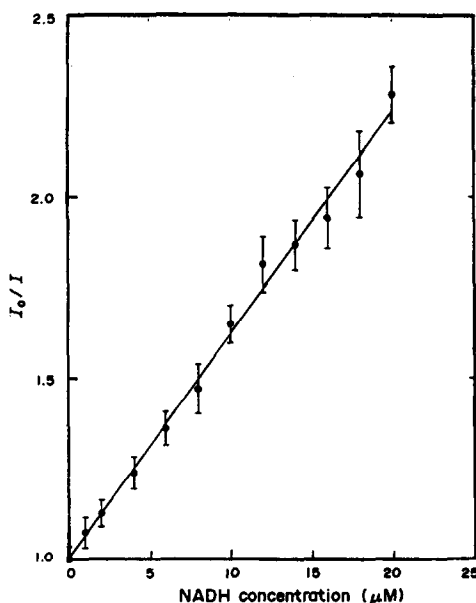


Fig. 3. Stern–Volmer plot for the quenching of thionine ($1\ \mu\text{M}$) fluorescence by NADH in a $0.1\ \text{pH}\ 7.0$ phosphate buffer.

where I_0 and I are the fluorescence intensities in the absence and presence of the quencher, respectively, K_{SV} is the Stern–Volmer quenching constant and $[Q]$ is the concentration of the quencher. Figure 3 shows the Stern–Volmer plot for NADH quenching data collected with an emission wavelength setting of $620\ \text{nm}$. A K_{SV} value of $6.2 (\pm 0.2) \times 10^4\ \text{M}^{-1}$ is obtained by linear regression analysis of these data.

The above information reveals that NADH is an extremely efficient quencher of thionine fluorescence. Half of the native fluorescence of thionine is quenched at an NADH concentration of only $16.1 (\pm 0.7)\ \mu\text{M}$. This half quenching concentration corresponds to a Stern–Volmer quenching constant of $6.2 (\pm 0.3) \times 10^4\ \text{M}^{-1}$ which matches the value given by the regression analysis. Clearly, a linear function accurately models these data. Of course, this linear relationship can be exploited for analytical purposes and the Stern–Volmer plot can be used as a calibration curve. From this calibration curve, the detection limit ($S/N=3$) for NADH by thionine fluorescence quenching is $1.6 (\pm 0.6)\ \mu\text{M}$.

The observed high efficiency of fluorescence quenching indicates that a process other than dynamic quenching is operative in this case. In dynamic quenching, the process is limited by molecular diffusion and the diffusion limited bimolecular collisional quenching rate constant (k_q) is related to the overall quenching constant (K_{SV}) by the following expression:

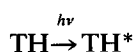
$$K_{\text{SV}} = k_q * \tau$$

where τ is the fluorescence decay time of the fluorophore in the absence of the quencher. A value for k_q can be calculated by using $6.2 (\pm 0.2) \times 10^4\ \text{M}^{-1}$ for K_{SV} and $350 (\pm 20) \times 10^{-12}\ \text{s}$ for τ [13]. The resulting value for k_q is $1.8 (\pm 0.1) \times 10^{14}\ \text{M}^{-1}\ \text{s}^{-1}$ which is three orders of magnitude higher than the rate constant for the reaction hydrogen and hydroxyl ions in water at 25°C ($1.4 \times 10^{11}\ \text{M}^{-1}\ \text{s}^{-1}$). Clearly, the bimolecular collision rate for the thionine–NADH pair cannot be faster than that for protons and hydroxide ions in water. This high value of k_q establishes that NADH quenching of thionine is not diffusion controlled and consequently, that dynamic quenching is not the principal mechanism for this process.

Results from a preliminary experiment suggest that the quenching mechanism is based on formation of a non-fluorescent exciplex between NADH and thionine. The intensity-time plot collected during this experiment is shown in Fig. 4. Initially, the native fluorescence intensity from a 1 μ M solution of thionine was monitored. At time "a", NADH was added to this solution such that the final NADH concentration was 12 μ M. As expected, the fluorescence intensity decreased with time owing to the quenching process. At time "b", a shutter between the sample and the excitation source was closed for 286 s. When the shutter was reopened, at time "c", the fluorescence intensity was higher relative to the intensity recorded immediately before the shutter was closed. The fluorescence intensity then decreased to the same value as when NADH was initially added.

The behaviour shown in Fig. 4 can be understood in terms of a photochemical reaction proposed by SHARMA [11] between the excited thionine (TH*) and NADH which results in a non-fluorescent (leuko/semi) thionine and NAD⁺. The reaction is given as below:

a. Light reaction



b. Dark reaction



Thus, at time "a" the above described photo-reaction between thionine and NADH is initiated. This results in a reduced population of the excited thionine and hence in a decrease in the fluorescence intensity at "b". When the shutter is closed, the non-fluorescent form of thionine recovers; thus following the opening of the shutter at time "c", higher intensity than at time "a" is observed. At this stage, due to the formation of the product, the concentration of quencher molecules (NADH) in the solution is expected to be less than that which was added at time "a". We expect the process to continue until all the NADH is consumed. At such a stage the thionine fluorescence will fully recover (some photobleaching of thionine is also expected), which was the case (see "e" in Fig. 4).

These observations indicate that the excited species of thionine must be present before quenching can occur and that the formation of a non-fluorescent exciplex is involved in the quenching process.

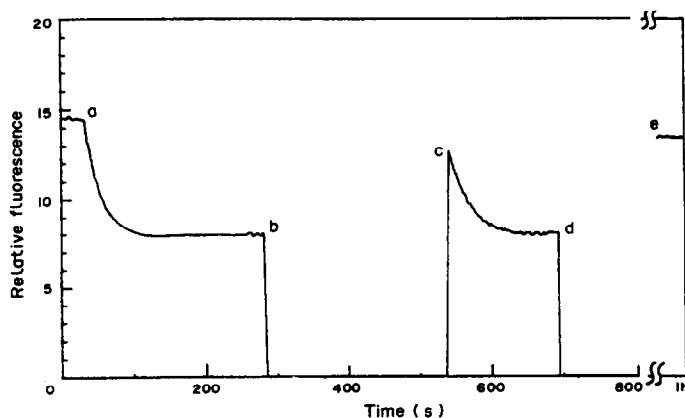


Fig. 4. Intensity-time plot for the quenching of thionine fluorescence by NADH, in 0.1 pH 7.0 phosphate buffer where at point "a" NADH is added to the solution, "b" a shutter between the excitation source and the sample is closed, "c" this shutter is reopened, and "d" is end of the experiment. The recovered fluorescence "e" after the total volume of thionine + NADH mixture is irradiated with the white light source for 1 h.

In a separate experiment, the absorption spectra of 1 μ M thionine was recorded both in the presence and absence of NADH. As expected, no changes in either of the NADH or of the thionine were observed. This confirms that no ground state interaction is involved. The above experiments and the high efficiency of this quenching process supports the conclusion drawn by SHARMA [11], which suggests that thionine undergoes a reversible photo-reduction by NADH resulting in NAD^+ .

The finding that NADH is an extremely efficient quencher of the fluorescence of thionine can have a great impact on analytical measurements in clinical settings. The possibility of making NADH measurements in whole blood matrix is of particular interest. Such measurements are not currently possible owing to the strong fluorescence and absorbance properties of the endogenous components in blood. Both the excitation and the emission properties of thionine are beyond those of the major constituents in whole blood. Thus, the measurement of thionine fluorescence, and consequently the quenching of this fluorescence by NADH, is possible. An important clinical application of such NADH measurements is in the quantification of enzyme activity for enzyme labels in immunoassays. Enzymes that either produce or consume NADH, such as glucose-6-phosphate dehydrogenase, are commonly used as labels in immunoassay procedures. A simple device to monitor NADH in whole blood based on NADH quenching of thionine could be used as the central detection scheme for a wide class of enzyme immunoassays [14].

CONCLUSIONS

The finding that thionine is efficiently quenched by NADH has several significant clinical and biotechnological applications. Optical properties of thionine are well suited for the use of simple, inexpensive and durable solid state optoelectronic components. In addition, the broad features of the excitation and emission spectra provide a great deal of flexibility in designing the optics for a particular application. These features suggest that a compact and inexpensive instrumentation can be readily developed for NADH measurements based on thionine fluorescence quenching. Corresponding work is in progress.

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