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ORIGINAL RESEARCH

Characteristics of endogenous flavin fluorescence of *Photobacterium leiognathi* luciferase and *Vibrio fischeri* NAD(P)H:FMN-oxidoreductase

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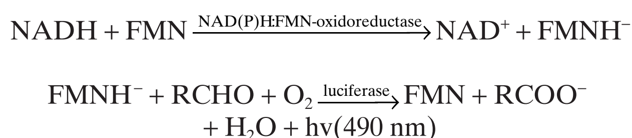
ABSTRACT: The bioluminescent bacterial enzyme system NAD(P)H:FMN-oxidoreductase–luciferase has been used as a test system for ecological monitoring. One of the modes to quench bioluminescence is the interaction of xenobiotics with the enzymes, which inhibit their activity. The use of endogenous flavin fluorescence for investigation of the interactions of non-fluorescent compounds with the bacterial luciferase from *Photobacterium leiognathi* and NAD(P)H:FMN-oxidoreductase from *Vibrio fischeri* has been proposed. Fluorescence spectroscopy methods have been used to study characteristics of endogenous flavin fluorescence (fluorophore lifetime, the rotational correlation time). The fluorescence anisotropy behaviour of FMN has been analysed and compared to that of the enzyme-bound flavin. The fluorescence characteristics of endogenous flavin of luciferase and NAD(P)H:FMN-oxidoreductase have been shown to be applicable in studying enzymes' interactions with non-fluorescent compounds. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: bacterial bioluminescence; flavin fluorescence

INTRODUCTION

Bacterial bioluminescence systems *in vitro* and *in vivo* are extensively used as bioassays in ecological monitoring of natural water bodies (1–5). The response of bioluminescence systems to the action of pollutants is of an integral characteristic, resulting from the multiple effect of exogenous compounds on bioluminescence (6). Therefore, it is important to investigate mechanisms of action of various chemical compounds on bioluminescence in order to predict the sensitivity of bioluminescence test systems to pollutants (7).

The bioassays are constructed on the basis of a bioluminescence system of coupled enzymatic reactions catalysed by NAD(P)H:FMN-oxidoreductase and bacterial luciferase:



$$NADH + FMN \xrightarrow{\text{NAD(P)H:FMN-oxidoreductase}} NAD^+ + FMNH^-$$

$$FMNH^- + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOO^- + H_2O + h\nu(490\text{ nm})$$

One of the mechanisms responsible for the influence of exogenous compounds on bioluminescence is their interaction with the enzymes, luciferase and NAD(P)H-oxidoreductase. Methods of fluorescence spectroscopy are widely used to study the dynamics of enzymes in different molecular environments (8). Sources of enzyme fluorescence can be both amino acid residues (tryptophan, tyrosine, phenylalanyl) and some co-factors (flavins, NADH). As a rule, an enzyme contains several fluorescent amino acid residues, which complicates the interpretation of experimental results. The co-factors are usually located near the active centres of enzymes. Their fluorescence can be used not only to study the dynamics of the enzyme but also to monitor changes in the active centre of the enzyme.

In this study we focused on the fluorescence properties of the oxidized flavin bound in luciferase and NAD(P)H:FMN-oxidoreductase.

It is known that the non-covalently bound endogenous flavin is present as a co-factor in the preparation of NAD(P)H:FMN-oxidoreductase isolated from *V. fischeri* (9–10). The luciferases of luminous bacteria are known to have affinity to the reaction product, FMN (11–12). Flavin is ideally suited for fluorescence studies aimed at obtaining the information about the enzyme dynamics and the flexibility of the active site (13–15).

We used the steady-state and time-resolved fluorescence and fluorescence anisotropy to gain information on the dynamic characteristics of flavin bound in preparations of luciferase and NAD(P)H:FMN-oxidoreductase. The data obtained on the differences in fluorescence anisotropy behaviour of flavin-bound and dissociated FMN would be useful to study the binding properties of FMN.

Comparison of the characteristics of the enzyme-bound flavin fluorescence will obviously contribute to understanding the process of interaction of exogenous molecules with the enzymes.

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MATERIALS AND METHODS

The lyophilized preparations of *P. leiognathi* luciferase (0.5 mg) and NAD(P)H:FMN-oxidoreductase–luciferase of *V. fischeri* (0.15 u. a. (unit of activity)) were produced by the laboratory of bacterial bioluminescence of the Institute of Biophysics SB RAS (Krasnoyarsk, Russia) (16). The FMN (Serva, USA) and 1,4-benzoquinone (Acros Organics, USA) were used. The assays were performed in the 0.05 mol/L phosphate buffer (pH 6.8) at 20°C.

Steady-state fluorescence

Excitation and emission spectra of FMN (10^{-6} mol/L), endogenous flavin in the preparations of luciferase and NAD(P)H:FMN-oxidoreductase were determined on a Fluorolog spectrofluorimeter. The wavelengths for excitation and emission were 450 nm and 540 nm, respectively.

Time-resolved fluorescence

Time-resolved fluorescence measurements were carried out using excitation with mode-locked lasers and detection with time-correlated photon counting electronics (17–18). The wavelengths for excitation and registration were 450 nm and 540 nm, respectively. Pinacyanol in ethanol was used as a reference compound to describe the instrument response (pinacyanol fluorescence decay is single-exponential, with a typical life-time of about 10 ps at 20°C).

One complete measurement consisted of measuring the polarized fluorescence from reference, sample, background and reference compound again. In that way an eventual temporal shift can easily be verified and corrected if necessary. The data analysis was performed on a personal computer, using the maximum entropy (19) method, the global analysis (20) approach, as well as a home-built program, able to fit models as a sum or distribution of exponential decays.

The fluorescence decay $I(t)$ and fluorescence anisotropy $r(t)$ decay curves were fitted by the sum of exponential terms:

$$I(t) = \sum_{i=1}^{N} p_i \exp(-t/\tau_i) \tag{1}$$

where τ_i and p_i are the fluorescence life-time and amplitude of the ith component, respectively, and N is the number of fluorescence components.

$$r(t) = \sum_{j=1}^{M} \beta_j \exp(-t/\phi_j) \tag{2}$$

where β_j and ϕ_j are the initial anisotropy and the rotational correlation time of the jth component of anisotropy, respectively, and M is the number of anisotropy components.

The fractional contribution of the ith component of fluorescence and jth component of anisotropy were calculated as:

$$f_i = \frac{p_i \cdot \tau_i}{\sum_j p_i \cdot \tau_i} \cdot 100\% \tag{3}$$

$$f_j = \frac{\phi_j \cdot \beta_j}{\sum_i \phi_j \cdot \beta_j} \cdot 100\% \tag{4}$$

The quality of the fit was estimated by the function of weighted residuals, autocorrelation function and the parameter χ^2. Both functions were randomly distributed around zero, and the values of χ^2 were minimized to 1.2.

RESULTS

Steady-state fluorescence

In this study, we considered the spectra of excitation and emission of endogenous flavin in the preparations of bioluminescence system enzymes. Fig. 1 shows the spectra of excitation (λ_{emis} = 540 nm) and emission (λ_{excit} = 450 nm) of the NAD(P)H:FMN-oxidoreductase and FMN preparations.

It is clearly seen (Fig. 1) that the emission spectra of FMN and NAD(P)H:FMN-oxidoreductase are almost identical. As to the excitation spectrum, the long-wave peak is less expressed for NAD(P)H:FMN-oxidoreductase than for FMN. Such changes in the spectra of excitation can be attributed to the deformation of the isoalloxazin fragment of the flavin bound to the enzyme. The spectral characteristics of the isoalloxazin fragment of the co-factor are known (21) to vary in different flavin-containing enzymes.

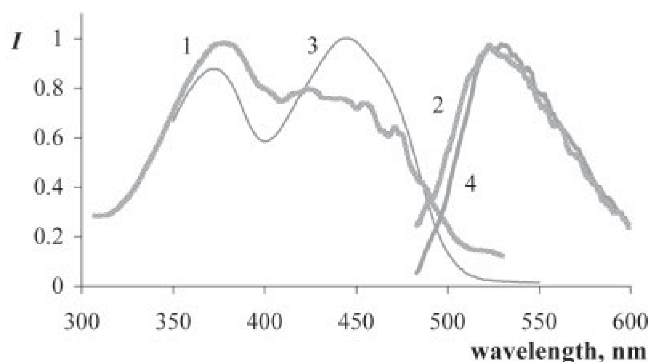

Figure 1. Excitation (λ_{emis} = 540 nm) and emission (λ_{excit} = 450 nm) spectra of preparations of NAD(P)H:FMN-oxidoreductase (1, 2, respectively) and FMN (3, 4, respectively).

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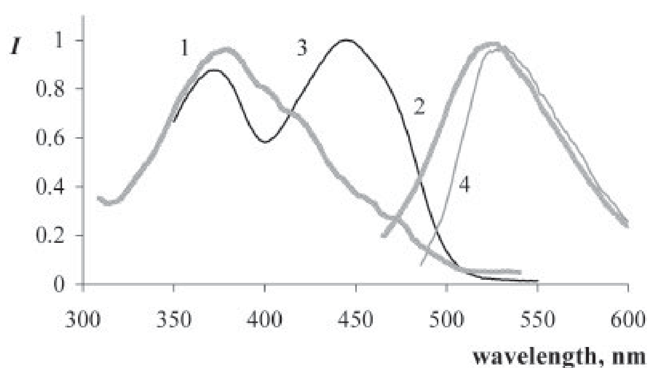

Figure 2. Excitation ($\lambda_{emis} = 540$ nm) and emission ($\lambda_{excit} = 450$ nm) spectra of preparations of luciferase (1, 2, respectively) and FMN (3, 4, respectively).

Similar studies were carried out using the luciferase preparation. Fig. 2 presents the excitation and emission spectra of luciferase. The analogous spectra of FMN are shown for comparison.

The emission spectra of FMN and luciferase agree closely, as in the case of NAD(P)H:FMN-oxidoreductase (Fig. 1). The disparity in the short-wave region of the spectrum may be due to the reabsorption of FMN luminescence in the region of overlapping of fluorescence and absorption spectra.

Similarly to the results of studying NAD(P)H:FMN-oxidoreductase (Fig. 1), common and distinctive features are observed in the excitation spectra of the FMN and luciferase preparations. The difference here is that in the case of luciferase, the spectrum maximum lying in the region of 450 nm is not pronounced in contrast to the analogous spectrum of FMN. Other researchers also reported the similar changes in the excitation spectrum of flavin bound to luciferase (11–12).

Thus, the comparison of the spectra of excitation ($\lambda_{emis} = 540$ nm) and emission ($\lambda_{excit} = 450$ nm) of the enzyme preparations—luciferase and NAD(P)H:FMN-oxidoreductase—with the corresponding spectra of FMN revealed the similarity of the ones. Our results show that under the abovementioned conditions for excitation of the enzymes, one can observe fluorescence of endogenous flavin.

Time-resolved fluorescence

Time-resolved fluorescence spectroscopy was used to study the characteristics of fluorescence decay of endogenous flavin in the preparations of luciferase and NAD(P)H:FMN-oxidoreductase.

Two exponents describe the fluorescence decay of FMN in a buffer solution (Table 1). The long-term component of 4.7 ns correlates with a FMN fluorescence lifetime in a solution and is similar to the values obtained by other researchers (14–15).

Four exponents at least were needed to describe the experimental decay of endogenous flavin fluorescence adequately, both for luciferase and NAD(P)H:FMN-oxidoreductase. The description of the decay in the enzyme preparations includes the components τ_1 and τ_2 characteristic of a free FMN. The values of τ_1 and τ_2 seem to be independent of the environment of the flavin chromophore. The emergence of the additional components, τ_3 and $\tau_{4,}$ seems to result from the interaction of endogenous flavin with the enzymes. The component τ_3 contributes considerably to the fluorescence decay and amounts to 2.9 ns, both in the luciferase case and in the case of NAD(P)H:FMN-oxidoreductase. The effect of the ultra-short component τ_4 is insignificant.

The comparison of fluorescence decay in free FMN with that in endogenous flavin in the preparations of the enzymes only characterises the state of chromophore in various environments. To obtain more detailed information on the enzyme dynamics, it is necessary to analyse fluorescence anisotropy.

Time-resolved fluorescence anisotropy

The curves of fluorescence anisotropy decay were built in accordance with the measurements of the kinetics of a polarized fluorescence of endogenous flavin under impulse excitation. Figs 3 and 4 show the results of studies of NAD(P)H:FMN-oxidoreductase, luciferase and the FMN solutions. Fluorescence anisotropy decay occurred at a much higher rate in free FMN than in endogenous flavin in the enzymes.

The fluorescence anisotropy decay of FMN in a solution was described with one exponent only (Table 2). The calculated rotational correlation time of the fluorophores, $\phi_{free} = 0.19$ ns, is in good agreement with the values reported (14–15).

The description of the endogenous flavin anisotropy decay, apart from the short-term component characterizing the free flavin, reveals the additional long-term components: $\phi_{bound} = 10.3$ ns in the case of NAD(P)H: FMN-oxidoreductase and $\phi_{bound} = 35.9$ ns for luciferase.

Table 1. Decay parameters for the fluorescence of free FMN, endogenous flavin in preparations of luciferase (L) and NAD(P)H:FMN-oxidoreductase (R)

Sample	τ_1(ns)	p_1	f_1(%)	τ_2(ns)	p_2	f_2(%)	τ_3(ns)	p_3	f_3(%)	τ_4(ns)	p_4	f_4(%)
FMN	0.70	0.05	0.7	4.7	0.95	99.3						
R	0.58	0.17	4.8	5.1	0.29	73	2.92	0.15	21.2	0.05	0.4	1
L	0.54	0.16	11.1	6.2	0.057	47.2	2.9	0.10	39.6	0.02	0.68	2.1

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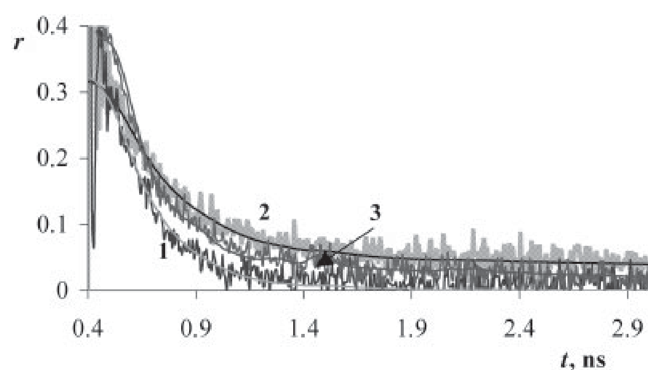

Figure 3. Fluorescence anisotropy decay at impulse excitation of: 1, FMN; 2, endogenous flavin in the preparation of NAD(P)H:FMN-oxidoreductase; 3, endogenous flavin in the preparation of NAD(P)H:FMN-oxidoreductase in the presence of 1,4-benzoquinone (8×10^{-3} M).

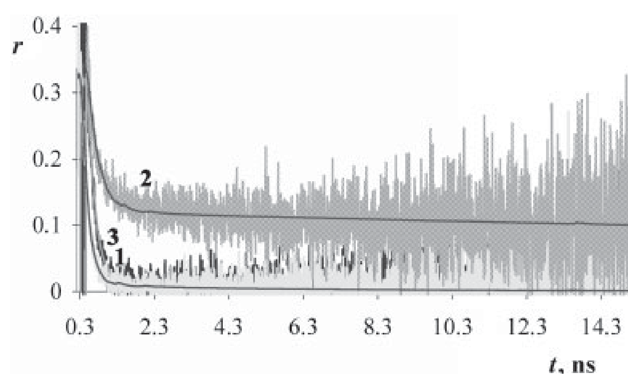

Figure 4. Fluorescence anisotropy decay at impulse excitation of: 1, FMN; 2, endogenous flavin in the preparation of luciferase; 3, endogenous flavin in the preparation of luciferase in the presence of 1,4-benzoquinone (8×10^{-3} M).

The values of these components are indicative of the interaction of endogenous flavin with the enzymes.

Our data show that the anisotropy decay in the enzyme preparations involves short and long components (Table 2). The rotational correlation time of the short component (ϕ_{free}) is comparable to that of free FMN.

The intrinsic rotational correlation times of the enzymes were estimated using the empirical formula (14) $\phi = 3.84 \times 10^{-4} \bullet M_r$ (M_r is molecular weight of the enzyme, kDa) as 10 ns for NAD(P)H:FMN-oxidoreductase and 32 ns for luciferase. In (22) the intrinsic rotational correlation time of luciferase was estimated experimentally as 34 ± 4 ns. These values are similar to the ϕ_{bound}-values experimentally obtained for the corresponding enzyme (Table 2), so it can be concluded that the long components under consideration correspond to those of the enzyme-bound flavin.

Hence, the calculated intrinsic rotational correlation times of the enzymes are comparable to the experimental long-term components of anisotropy decay in endogenous flavin fluorescence (Table 2), indicating that the analysed endogenous flavin is bound to luciferase and NAD(P)H:FMN-oxidoreductase in the investigated enzyme preparations.

It should be stressed that the fractional contribution of long-term components is much larger than that of the short-term ones describing the behaviour of flavin molecules in a free state (87.4% and 12.6% for NAD(P)H:FMN-oxidoreductase, and 97.8% and 2.2% for luciferase, respectively). The data obtained suggest that the major part of endogenous flavin is bound with the enzymes.

The next stage was addition of 1,4-benzoquinone to the enzymes. The result was a decrease in fluorescence anisotropy decay of endogenous flavin in NAD(P)H:FMN-oxidoreductase (Fig. 3). The anisotropy decay of endogenous flavin was described by two components as in the case of the enzyme without quinone. The ϕ_{bound} dropped to 3.3 ns. As this took place, the contribution of the long component (f_{bound}) decreased, while that of the short component (f_{free}) increased in the presence of 1,4-benzoquinone. This indicated a loosening of binding (8) of endogenous flavin to the enzyme and a rise in free FMN concentration.

The fluorescence anisotropy decay of endogenous flavin in luciferase preparation in the presence of 1,4-benzoquinone was actually the same as that of free FMN (Fig. 4). The decay was described by a short component (ϕ_{free}) only (Table 2).

Thus, the changes in the long-term components of rotational correlation time of endogenous flavin fluorescence anisotropy in the presence of 1,4-benzoquinone suggest a conclusion that quinone interacts with the bioluminescent system enzymes—luciferase and NAD(P)H:FMN-oxidoreductase. The result of the

Table 2. Decay parameters for the fluorescence anisotropy of free FMN, endogenous flavin in preparations of luciferase (L) and NAD(P)H:FMN-oxidoreductase (R) in the presence and in the absence of 1,4-benzoquinone

Sample	ϕ_{free}(ns)	β_{free}	f_{free}(%)	ϕ_{bound}(ns)	β_{bound}	f_{bound}(%)
FMN	0.19 ± 0.01	0.34	100	–	–	–
R	0.26 ± 0.01	0.27	13	10 ± 1	0.05	87
L	0.24 ± 0.01	0.22	2.2	36 ± 4	0.063	98
R + 1,4-benzoquinone	0.15 ± 0.01	0.36	28	3.3 ± 0.4	0.04	72
L + 1,4-benzoquinone	0.14 ± 0.01	0.37	100	–	–	–

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interaction is the loosening of binding of endogenous flavin to the enzymes.

CONCLUSION

Thus, the study determined the characteristics of fluorescence of endogenous flavin in luciferase and NAD(P)H:FMN-oxidoreductase preparations. As protein-bound and free FMN were compared, differences in their fluorescence behaviour were used to study the association process and the binding properties of FMN. Moreover, comparison of characteristics of enzyme-bound flavin fluorescence anisotropy, such as fluorophore rotational correlation time (ϕ_{bound}) and its fractional contribution (f_{bound}), in the presence and absence of exogenous non-fluorescent compounds can be one of the steps on the way to understanding the dynamics of the enzyme in different molecular environment and the process of interaction of exogenous molecules with enzymes.

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