

Binding of Ethidium to Yeast tRNA^{Phe} : A New Perspective on an Old Bromide

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We have reinvestigated the binding of ethidium bromide (EB) to yeast tRNA^{Phe} using frequency domain fluorometry and Global Analysis. Previous fluorescence investigations of EB – tRNA interactions, carried out for more than 30 years, have indicated a “strong” binding site with a lifetime near 26 ns and one or more “weak, non-specific” binding sites with reduced lifetimes. In our study, under specific conditions in which only one EB is bound, a fluorescence lifetime of 27 ns was obtained. However, as the EB/tRNA ratio increased, shorter lifetime components appeared. Global Analysis of the lifetime data was consistent with a model in which the second EB molecule bound has a lifetime of only 5.4 ns. Global Analysis also indicated that this second binding event leads to a reduction in the lifetime of the first EB bound, namely from 27 ns to 17.7 ns. The lifetime decrease associated with the “strong” binding site could be due to a quenching process arising either from energy transfer between EB molecules or from alterations in the conformation of the tRNA, or both. These results are considered in light of recent NMR observations on an EB/tRNA system. We also investigated the effect of ionic strength on the lifetime and relative affinities of these two binding components and found that NaCl levels up to 900 mM did not significantly affect the results.

6.1 Overview

Spectroscopic investigations of ethidium bromide (EB) (CAS Number/Name: 1239-45-8/Phenanthridinium-3,8-diamino-5-ethyl-6-phenyl-bromide) interactions with transferRNA (tRNA) have been carried out for more than 30 years [1–21]. The spectroscopic techniques utilized include NMR, X-ray and optical methods such as circular dichroism, absorption and fluorescence. The consensus of these studies is that one or more EB intercalates into the acceptor stem of tRNA [1, 4, 6, 7, 19]. X-ray [8] and NMR [4, 6] studies have furthermore identified a specific region of the acceptor stem of tRNA with the “strong” binding site.

We reinvestigated the interaction of EB with yeast tRNA^{Phe} using time-resolved fluorescence, specifically utilizing multifrequency phase and modulation fluorometry coupled with Global Analysis [22]. This approach allowed us to resolve several classes of fluorescence lifetimes, that vary in intensity as the EB/tRNA ratio is changed. Global Analysis of the intensity decay data supports a model in which the lifetime of the EB bound to the “strong” binding site is significantly reduced upon binding of subsequent EB molecules to the EB/tRNA complex.

6.2 Experimental

6.2.1 Sample Preparation

Yeast tRNA^{Phe} was obtained from Sigma (St. Louis, MO) and used without further purification. EB was obtained from Molecular Probes (Eugene, OR). Unless otherwise indicated, the buffer utilized was pH 7, 20 mM HEPES, 2 mM MgCl₂, 0.1 mM EDTA, 100 mM KCl.

6.2.2 Multifrequency Phase and Modulation Fluorometry

Intensity decay data were obtained using an ISS K2 multifrequency phase and modulation fluorometer utilizing a Spectra-Physics model 2045 Argon-Ion laser as the excitation source. Samples were excited at 514 nm and emission >550 nm was viewed through a Schott OG87 cut-on filter. Phase and modulation data were collected over a frequency range of 0.8 MHz to 80 MHz and analyzed using Global software. Fig. 6.1 shows phase and modulation curves for four of the seven EB/tRNA ratios utilized (three of the data sets used in the analysis are omitted for clarity). The raw data clearly show changes in the lifetime properties as the EB/tRNA ratio increases.

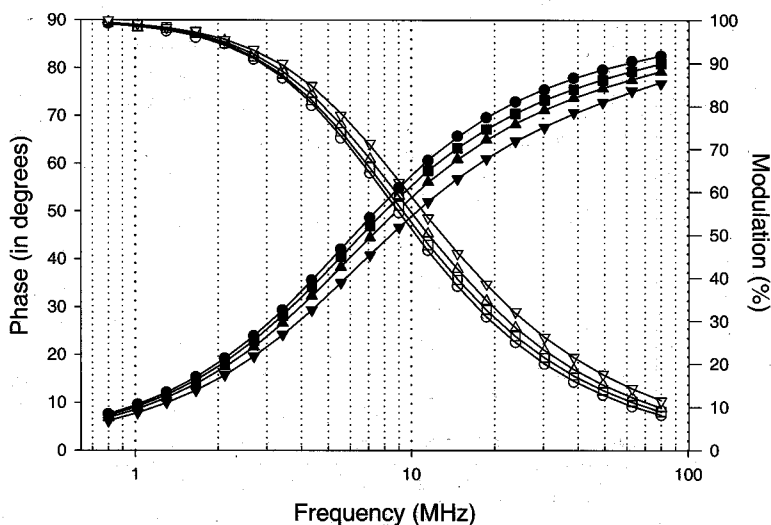


Fig. 6.1 Multifrequency phase (filled symbols) and modulation (open symbols) data for EB/tRNA ratios of 0.27 (circles), 1.34 (squares), 2.41 (triangles) and 4.05 (inverted triangles)

6.2.3 Three Component Analysis

Our initial approach to analyzing this lifetime data was to use three components: one corresponding to free EB, plus two other discrete exponentials. Figs. 6.2 and 6.3 show two such lifetime analyses of this data set – lifetime components and fractional intensities are displayed as a function of the EB/tRNA ratio. In the first lifetime analysis (Fig. 6.2) a short (1.8 ns) component corresponding to free EB is fixed but the other two components are free to vary. One notes that lifetime 2 fluctuates between ~ 5.7 – 7.9 ns whereas lifetime 3 decreases monotonically from about 27 ns to about 24 ns. In the second analysis (Fig. 6.3), lifetime 2 is linked (but not fixed) between the data sets and is about 7.2 ns; in this case lifetime 3 still decreases monotonically from ~ 27 ns to ~ 24 ns.

In both cases, the data fits appear good as judged by the reduced chi-square values which were both ~ 0.3 (assuming standard errors of 0.2° in phase angle and 0.004 in the modulation ratio).

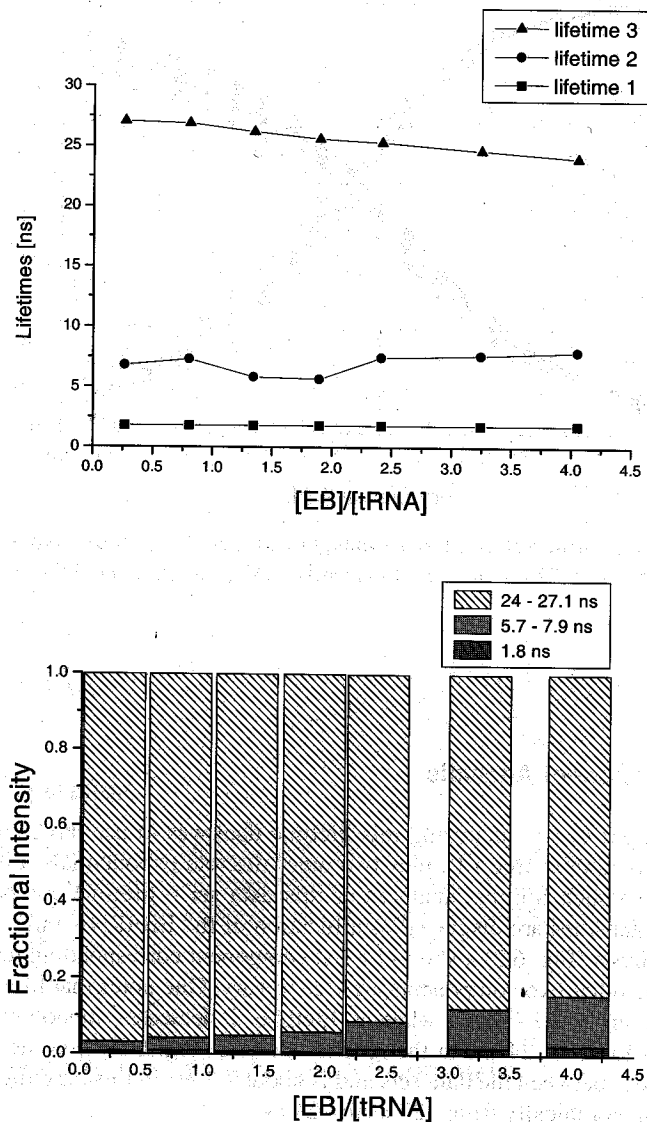


Fig. 6.2. Three component analysis of lifetime titration data. No components are linked but the 1.8 ns component is fixed

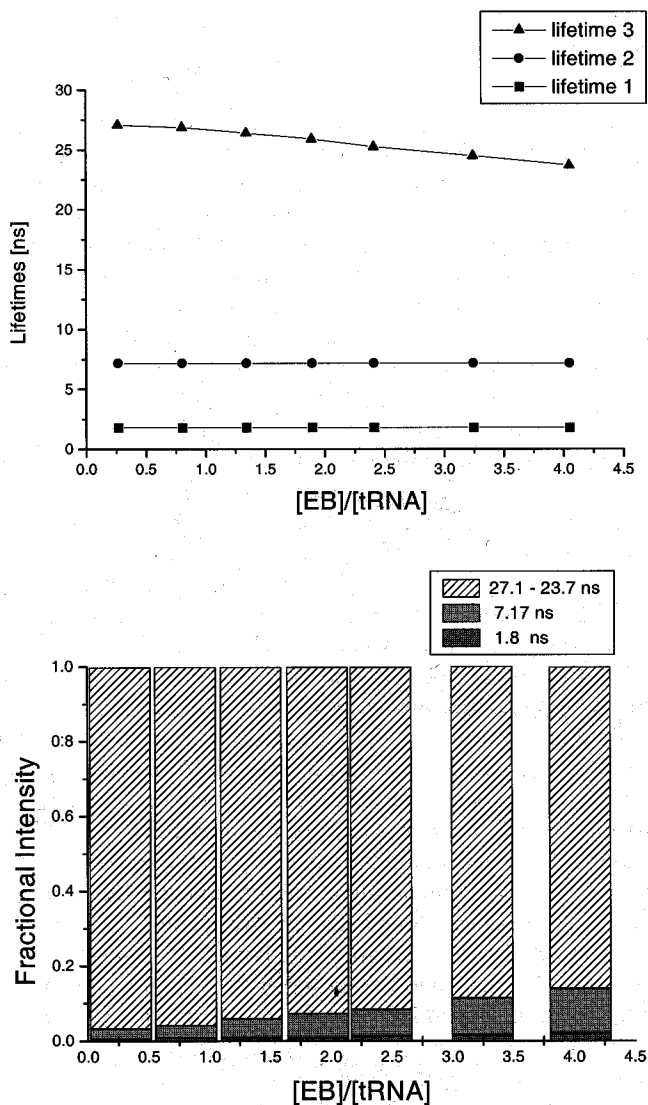


Fig. 6.3. Three component analysis of lifetime titration data. The intermediate lifetime component is linked and the 1.8 ns component is fixed

6.3 New Analysis

As mentioned above, the quality of the data fit in both of the preceding three-component analyses was good. However, except for fixing one component at 1.8 ns which corresponds to the known lifetime of free EB, these analyses did not take into account the nature of the multiple equilibria one expects in such a system. Specifically, as the titration progresses one expects some of the tRNA molecules to have only one EB bound and the lifetime of this species should be invariant. We thus carried out a three component analysis, with the long lifetime component linked throughout all data sets, and with a 1.8 ns component fixed, but with the second, shorter lifetime component free to vary and with the fractional intensities associated with each component free to vary. In this scenario, lifetime 2 increased from ~3.2 to 11.4 ns and the quality of the fit was reduced (chi-square ~0.8).

We then carried out a four-component analysis (Fig. 6.4) in which lifetime 1 was fixed to 1.8 ns (corresponding to free EB), while lifetimes 2, 3 and 4 were free to vary but were linked across the data sets; the fractional intensities associated with each component were free to vary. The quality of the fit to this model was very good (chi-square ~0.25). In this analysis, the resolved lifetime components are 5.4 ns, 17.7 ns and 27 ns, with standard errors of approximately 10–15% in each case. The fractional intensity corresponding to the long (27 ns) component decreases as the EB/tRNA ratio increases while the fractional intensities corresponding to the other three components all increase (Fig. 6.4).

We note that one can also analyze time-resolved data in terms of the pre-exponential factors as opposed to fractional intensities. To relate the pre-exponential factors to the actual number of different emitting molecules, however, requires knowledge of the relative quantum yields of the molecular species, at the excitation wavelength utilized and the emission wavelengths observed. In the present case, we have no information regarding the absorption and emission spectra of the 5.4 ns component. Nor do we know if the spectra for the longer lifetime component remain the same after the second EB is bound. We note that the excitation wavelength utilized (514 nm) and the emission wavelengths observed (>550 nm) were chosen to weigh the bound EB over the free EB to facilitate resolution of the bound EB lifetimes.

We determined, however, upon excitation at 501 nm (the approximate isosbestic wavelength for free and bound EB) and observing all emission >530 nm, that the enhancement in the yield of the EB bound was approximately 9-fold. This enhancement was measured using a large excess of tRNA compared to the EB concentration so that no tRNA should have more than one EB bound. We note this value to be less than one might expect from the ratio of the free and bound lifetimes (e.g., 27 ns/1.8 ns = 15). Given the fact that the absorption and emission spectra of free and bound EB differ substantially, however, there is no *a priori* reason to expect the ratio between lifetimes and quantum yields to be identical.

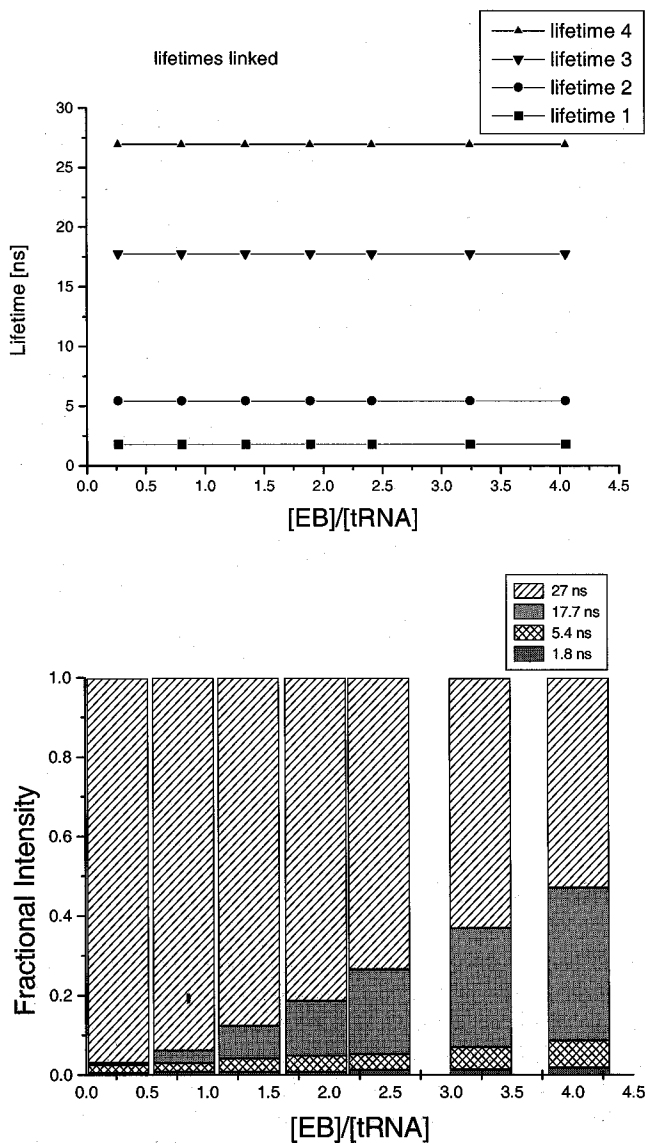


Fig. 6.4. Four component analysis of lifetime titration data. All lifetime components are linked (the 1.8 ns component is fixed) but the fractional intensities are free to vary

6.4 The Model

Our interpretation of this analysis is that EB in the “strong” binding site (which has a dissociation constant of $\sim 1\text{--}2\ \mu\text{M}$ based on steady-state titration data (not shown) has a lifetime of 27 ns if the “weak” binding site is not occupied. The lifetime of EB bound to the “weak” binding site (which has a dissociation constant in the range of $20\text{--}40\ \mu\text{M}$; data not shown) is 5.4 ns. However, when the “weak” binding site is occupied, the lifetime of EB in the “strong” binding site is reduced to 17.7 ns. This model is illustrated in Fig. 6.5.

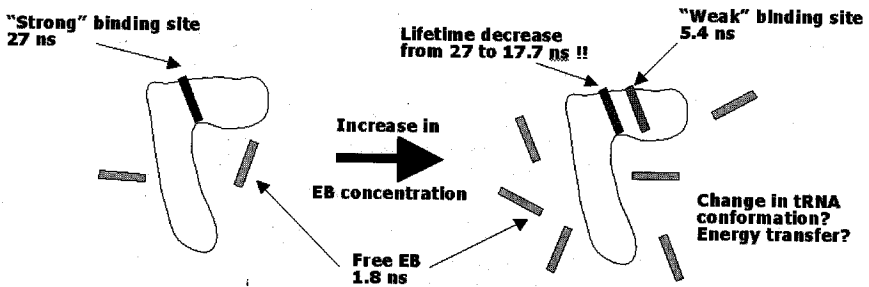


Fig. 6.5. Schematic model depicting the various lifetimes of EB bound to tRNA

6.5 Effect of Ionic Strength

It has previously been reported that increasing ionic strength, specifically NaCl, abolishes non-intercalative interactions between EB and tRNA [19]. If the 5.4 ns component is due to EB bound in a non-intercalative manner, one would expect that increasing ionic strength would reduce the relative amount of this component. Therefore, we studied the effect of varying levels of NaCl on the lifetime data at an EB/tRNA ratio of 4. As shown in Fig. 6.6, the effect of NaCl up to 900 mM was negligible as regards the actual lifetimes recovered and the relative proportions of the lifetime components.

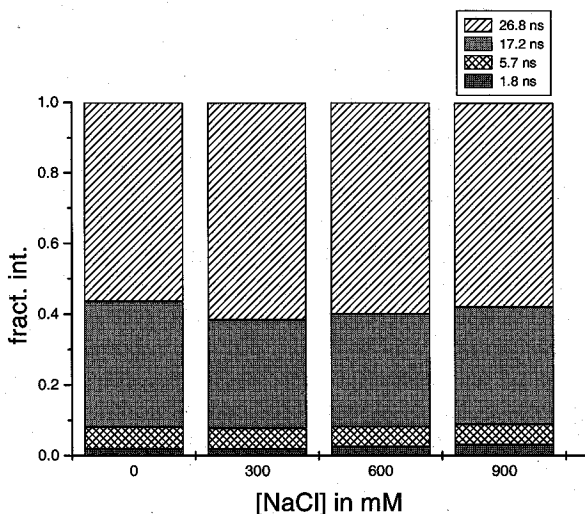


Fig. 6.6. Results of four component linked lifetime analysis of 100 μ M EB and 400 μ M tRNA in standard buffer in the presence of increasing NaCl concentrations

6.6 Conclusions

Previous measurements of the fluorescence lifetime of EB associated with tRNA (at low EB/tRNA) ratios, generally found values in the range of 26–28 ns [5, 7, 18, 21, 23]. Our present results are consistent with these earlier observations, i.e., at low EB/tRNA ratios we find a single exponential decay of 27 ns. As the EB/tRNA ratio increases we note a distinct decrease in the “average” lifetime of the system. Global Analysis of multiple data sets, obtained at increasing EB/tRNA ratios, supports a model in which binding of additional EB leads to a decrease of the lifetime of the EB in the “strong” binding site. Specifically, the lifetime of the initially bound EB decreases from 27 ns to 17.7 ns.

Proton NMR experiments of Jones and Kearns [6] suggested that the “strong” EB binding site was located near base pairs AU6 and AU7 in the amino acceptor stem. More recently, Chu et al., [4] used 19F NMR (by incorporation of 5-fluorouracil into specific locations in the tRNA) and proton NMR to study EB binding to *Escherichia coli* tRNA^{Val}. They also report the “strong” EB binding to be between base pairs A6:U67 and U7:A66, in agreement with the observations of Jones and Kearns. Chu et al. [4] also found evidence for a second, weaker EB binding site in the amino acceptor stem, near U4:A69 and G5:C68. This result suggests the two EB binding sites may be quite close to one another. Hence, the decrease we observed in the lifetime (27 ns to 17.7 ns) of EB bound to the

“strong” site could be due either to a change in the tRNA conformation near the “strong” binding site or energy transfer from EB in the “strong” site to EB in the “weak” site. A change in the conformation of the tRNA near the “strong” binding site could, for example, increase the accessibility of water to the excited EB, which could decrease the quantum yield [9].

Clearly, these results suggest the analysis and interpretation of steady-state fluorescence titration data on this system must take into account the decrease in the fluorescence enhancement of the first EB bound when the second site is occupied. Additionally, the very small influence of salt concentration on the “weak” binding site is interesting since it suggests the 5.4 ns component is due to an intercalated, as opposed to a non-intercalative, bound EB. Our results are also consistent with the observations of Ghribi et al., [20] who reported that at higher ionic strengths tRNA bound only a small number of EB molecules. Finally, we wish to point out that analysis of steady-state fluorescence titrations on EB/tRNA systems must take into account the decrease in the quantum yield of the first bound EB as subsequent EB molecules bind.

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