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Luminescence of peptide-bound terbium ions

Determination of binding constants

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Luminescence of Tb^{3+} ions bound to a calmodulin fragment has been studied. It is shown that during their lifetime excited ions dissociate from the peptide. If concentration of free peptide is high enough they can be coordinated again. As a consequence, observed terbium luminescence lifetime and intensity depends not only on binding equilibrium, but also on concentration of free peptide molecules. In such a system terbium binding constant cannot be correctly determined by simple steady-state measurements of luminescence intensities. Instead, terbium luminescence decay curves measured at various peptide concentrations must be analysed. Such an analysis has been made for a fragment of the IIIrd calcium binding domain of rat testis calmodulin. Rate constant of terbium association and the equilibrium binding constant corresponding to the best fit of theoretical functions to experimental points have been determined.

Terbium binding to peptide; Terbium luminescence; Calmodulin fragment

1. INTRODUCTION

Terbium ion Tb^{3+} was found to bind to many calcium binding proteins [1] and shown, in several cases, to occupy the same binding sites as Ca^{2+} ion [2]. Both ions have almost the same radius and the same coordination properties [1]. Advantages of using terbium for investigation of calcium binding sites come fromits higher charge, resulting in stronger binding, and from its luminescence properties. The luminescence of free terbium in water solution is weak (because of weak absorption bands and strong quenching by water OH groups [3]), but often enhanced upon binding [1]. This allows for easy monitoring of binding equilibrium and determination of binding constants [4-7]. Terbium is also used for determination of calcium binding constants, by monitoring displacement of bound terbium ions by large excess of calcium [4,5,7]. The luminescence enhancement of Tb^{3+} is caused by two mechanisms: (i) because bound terbium is shielded from water OH groups, its quantum yield increases with accompanying increase of its luminescence lifetime (from 0.4 ms in water [3] to ca. 1.3 ms in proteins [3,8]); (ii) terbium is a good acceptor for excitation energy transfer from nearby aromatic amino acid residues often encountered in sequences of binding loops [9]. This results in much more effective excitation.

An all papers published so far terbium binding constants have been determined assuming that measured luminescence signal can be described by the following equation:

$$S = \text{const}'[M_b] + \text{const}''[M_f] \quad (1)$$

where $[M_b]$ and $[M_f]$ are the concentrations of bound and free metal ions, respectively, and const' >> const''.

In this work we show that this assumption is not correct and can lead to substantial errors in determination of Tb^{3+} binding constants.

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2. MATERIALS AND METHODS

2.1. *Peptide*

A fragment of the IIIrd calcium binding domain of rat testis calmodulin was synthesised by the conventional Merrifield method and purified by reversed phase HPLC. Its sequence, containing the full Ca^{2+} binding loop (residues 13-24), is the following:

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  1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17
AcSerGluGluGluIleArgGluAlaPheArgValPheAspLysAspGlyAsp-
 18 19 20 21 22 23 24
GlyTyrIleSerAlaAlaGluNH2
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A stock solution of this peptide in water (0.53 mM) was prepared. Its concentration was determined spectrophotometrically on the basis of tyrosine absorption. Samples for titration experiments were prepared by diluting the stock solution 107 times in 20 mM cacodylic buffer, 0.1 M NaCl, pH 6.9 in a 10 mm quartz cuvette. Prior to titration nitrogen was bubbled through the sample.

2.2. *Terbium*

Terbium chloride hexahydrate from Aldrich (99.999%) was dissolved in 20 mM cacodylic buffer, pH 6.0, 0.1 M NaCl. Terbium concentration was determined by EDTA titration using Xylenol orange as an endpoint indicator [10]. Terbium solution used for ex-

periments ([Tb] = 0.6 mM, pH 6.9) was prepared from the stock solution by dilution in 20 mM cacodylic buffer, 0.1 M NaCl.

2.3. *Spectrofluorimeter*

Terbium and peptide Tyr-19 luminescence were measured using a Cobrabid (Opole, Poland) spectrofluorimeter modified in our laboratory. It consisted of a UV light source, an input monochromator, a beam splitter, a reference photomultiplier, a thermostatted sample-holder, an output monochromator, and an output photomultiplier. As the light source a high-pressure Hg lamp was used. The beam splitter placed between the input double prism monochromator and a sample allowed to monitor excitation light intensity with the use of rhodamine photon-counting solution and the reference photomultiplier (EMI 9558B). Amplified and digitised signals from this photomultiplier were computer sampled with 100 Hz frequency. Luminescence emitted by the sample was viewed perpendicularly to the excitation beam. After passing through the output grating monochromator it was focused on a photocathode of the output photomultiplier (RCA 8850) equipped with a magnetic lens and cooled to −25°C. Amplified single photon pulses from the photomultiplier have been counted and the state of the counter has been read and memorized by a computer. Luminescence intensities were corrected for excitation light fluctuations using the signal of reference photomultiplier integrated over the time of measurement. Excitation wavelength of 280 nm (10 nm bandwidth) allowed for efficient excitation of peptide-bound terbium *via* energy transfer from Tyr-19 residue [4–7]. Only minor excitation of free terbium was observed. The detection wavelength of terbium luminescence was 545 nm.

For luminescence decay measurements the mechanical light chopper has been used, giving 5 ms periods of excitation and 5 ms dark periods of fluorescence decay, with the shutting-off time of 150 μs. Also the output monochromator was substituted by a filter cutting off UV light (GG10, Schott, Jena). The luminescence signal was sampled in 17 μs intervals.

Sample absorbance was kept below 0.09. All measurements were made at 25°C.

2.4. *Least-squares fitting*

To fit theoretical model to experimental data two programs for least-squares fitting were used. One, based on the simplex method [11], for an initial step of fitting procedure, and the second, using gradient algorithm [12], for a final step.

The minimized function F was the sum of the weighted squares of deviations of experimental values of fluorescence signal from theoretically calculated ones for all points in the set of decay curves.

$$F = \sum_i (S_i^{exp} - S_i^{calc})^2 / S_i^{exp}$$

The error limits for parameter values were obtained by fixing one parameter, minimizing F with other parameters free to change, fixing the parameter at a new value, minimizing F, and so on. The values of parameter which doubled the value of function F has been taken as the borders of confidence of our fit.

3. RESULTS

We have measured terbium luminescence intensity and lifetime, as well as peptide tyrosine fluorescence intensity upon titration of the peptide by terbium. When terbium is added to a peptide solution, strong terbium luminescence is observed, accompanied by decrease of peptide tyrosine fluorescence. Titration curves of both signals show saturation characteristic for terbium binding (not shown). Nevertheless, measurements of terbium luminescence lifetimes gave unexpected results. When a large excess of terbium is present in solution (100 μM terbium vs 5 μM peptide) luminescence lifetime

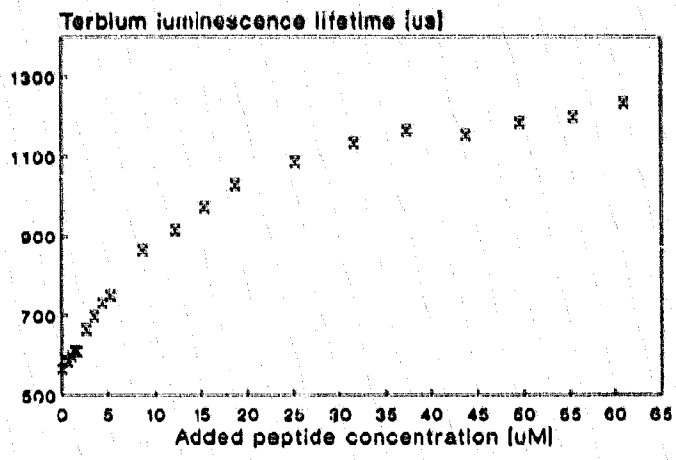

Fig. 1. Changes of terbium luminescence lifetime upon titration of 5 μM Tb^{3+} by peptide AcSEEEIREAFRVFDKDGDGYISAAENH$_2$ in 20 mM cacodylic buffer, 0.1 M NaCl, pH 6.9, temp. 25°C.

(599 μs) is practically equal to the value measured independently for free terbium in buffer solution (600 μs) and not to that of terbium bound to proteins (1.3 ms) [3,8], although the observed luminescence comes almost entirely from peptide-bound ions (more than 95% of the total signal). Therefore, the increase of luminescence intensity of terbium upon binding to peptide originates only from more efficient excitation, but quenching processes are not affected.

Two hypotheses could rationalize this observation. (i) A terbium ion bound to peptide is not shielded from water OH groups – the amount of water molecules in the first hydration sphere of the ion does not change upon binding. (ii) A bound terbium ion excited *via* tyrosine dissociates instantaneously to the solution. So, in practice, only the luminescence originating from free ions is observed. In other words, the rate constant of

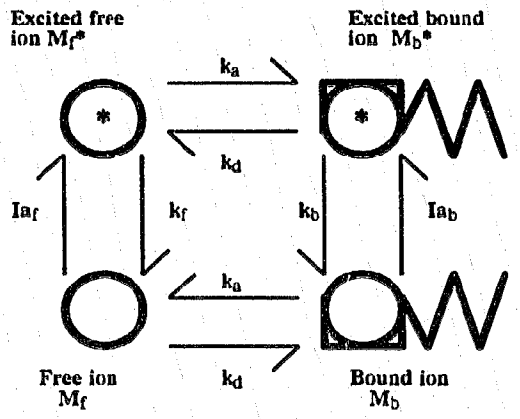

Fig. 2. Kinetic processes in solution containing metal ions and metal-binding peptide molecules exposed to excitation light of intensity I. It is assumed that association and dissociation rate constants k_a and k_d, respectively, are the same for excited and non-excited ions. k_b,k_f = decay rate constants of bound and free excited ions, respectively. a_b,a_f = excitation efficiencies of bound and free ions, respectively (see text).

ion dissociation is much greater than that of its fluorescence decay. This last value, for terbium bound to a protein, equals $1/1.3$ ms $= 770$ s^{-1}.

The second hypothesis has been confirmed by measurements of luminescence decay of 5 μM terbium in the presence of increasing peptide concentration. The decays can be approximated by monoexponential functions. The best-fit luminescence lifetimes are shown in Fig. 1. They increase with peptide concentration and approach the value characteristic for protein-coordinated terbium ions. This result is clearly incompatible with the first hypothesis, but can be explained by the second one. As the concentration of free peptide molecules increases, the excited terbium ions dissociated from the complex are trapped again with increasing rate. Finally, the observed luminescence comes practically from bound terbium, even if dissociation probability is high.

In the studied system (Fig. 2) concentrations of excited terbium ions, bound and free, change with time according to the following equations:

$$d[M_b^*]/dt = I_{a_b}[M_b] - (k_d + k_b)[M_b^*] + k_a[P_f][M_f^*] \quad (2)$$

$$d[M_f^*]/dt = I_{a_f}[M_f] + k_d[M_b^*] - (k_a[P_f] + k_f)[M_f^*]$$

where the symbols are defined in Fig. 2 and the rate constants of dissociation and association are assumed to be the same for excited and non-excited ions. Upon binding to a protein, both absorption and emission spectra of terbium ions are known not to change in any significant way. In such a case the Förster cycle analysis [13] shows that the binding constants should not change upon excitation. For the same reason it can be assumed that the luminescence rate constants for free and bound terbium are the same, so that the measured luminescence signal is strictly proportional to the total concentration of excited ions:

$$S(t) = \text{const}\,([M_f^*(t)] + [M_b^*(t)]) \quad (3)$$

In steady state $d[M_f^*]/dt = d[M_b^*]/dt = 0$ and $I = \text{const}$. Therefore

$$[M_b^*] = I(a_b[M_b](k_f + k_a[P_f]) + a_f[M_f]k_a[P_f])/D$$

$$[M_f^*] = I(a_b[M_b]k_d + a_f[M_f](k_d + k_b))/D \quad (4)$$

where $D = (k_d + k_b)(k_a[P_f] + k_f) - k_a[P_f]k_d$

Thus, from (3) and (4):

$$S = \text{const}\,\{a_b[M_b](k_f + k_a[P_f] + k_d + a_f[M_f](k_b + k_a[P_f] + k_d)\}/D \quad (5)$$

This expression takes a form corresponding to Eqn 1 for low concentrations of free peptide, so that $k_a[P_f] << k_f$. It can be shown that Eqn 1 is also a good approximation of Eqn 5 if $k_d << k_b$. Nevertheless, in general, the luminescence signal depends not only on $[M_b]$ and $[M_f]$, but also on free peptide concentration $[P_f]$. The parameters in Eqn 5 cannot be determined by simple measurements of luminescence intensity in titration experiments. Even discrimination between the systems for which Eqn 1 is and is not applicable cannot be made on the ground of steady state measurements. In fact, for the system studied in this work it is possible to obtain a perfect fit of Eqn 1 to terbium luminescence intensities, measured at increasing peptide concentration. The best-fit binding constant equals 4.7×10^4 M^{-1}. Therefore, the only procedure that makes it possible to determine the parameters of Eqn 5 and calculate the binding constant in the correct way is an analysis of luminescence decay curves measured at various peptide concentrations.

With the assumption that the fraction of excited ions is very small, so that $[M_b]$ and $[M_f]$ are time-independent variables, one can obtain a general solution of a set of linear differential Eqns 2. The matrix characteristic for this set of equations is as follows:

$$T = \begin{bmatrix} -(k_d + k_b) & k_a[P_f] \\ k_d & -(k_a[P_f] + k_f) \end{bmatrix}$$

The general solution in the case of luminescence decay period ($I = 0$) is:

$$[M_f^*(t)] = A(1,1)\exp(r_1 t) + A(1,2)\exp(r_2 t)$$

$$[M_b^*(t)] = A(2,1)\exp(r_1 t) + A(2,2)\exp(r_2 t)$$

where r_i are the eigenvalues of the matrix T and $A(i,k)$ are the elements of the eigenvectors of this matrix.

To calculate the numerical values of eigenvectors we have to know the initial conditions, i.e. the values of concentrations $[M_f^*]$ and $[M_b^*]$ in the beginning of the decay measurement – $[M_f^*(0)]$ and $[M_b^*(0)]$. If the excitation beam is cut off instantaneously and the excitation period is very long as compared with the luminescence decay time the initial conditions are determined by steady state conditions given by Eqn 4. In the apparatus used in this work both the excitation period (5 ms) and the shutting-off time (150 μs) are comparable with the observed decay times (600–1300 μs). So, the values of $[M_f^*(0)]$ and $[M_b^*(0)]$ have been calculated using the solutions of Eqns 2 for $I_0 > 0$ and assuming that the light intensity is constant during the excitation $I = I_0$ and drops linearly with time during the shutting-off period $I = (1 - t/150\ \mu s)I_0$. This last relation was found by measurements of scattered light intensity.

The decay curves of 255 points each have been measured for solutions containing 5 μM terbium and variable peptide concentrations from 0 up to 62.5 μM. Theoretical decay curves have been fitted to the whole set of 5100 experimental points. With the following

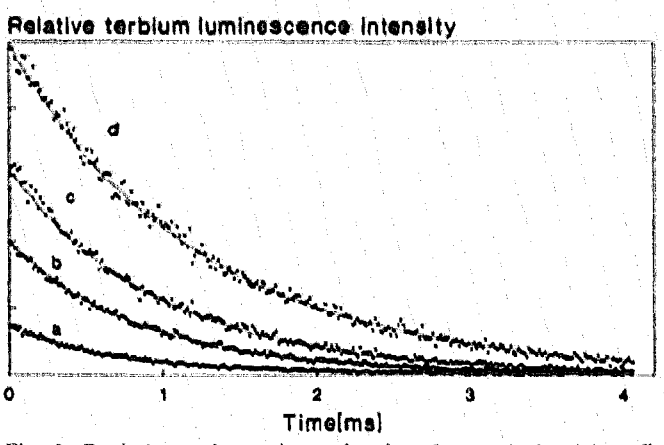

Fig. 3. Deviations of experimental points from calculated best-fit curves of terbium luminescence decay at constant terbium concentration of 5 μM and four chosen peptide concentrations: 3.5 μM (a), 12.1 μM (b), 25.2 μM (c) and 61 μM (d).

parameters: $k_a = 1\times10^9\ M^{-1}\cdot s^{-1}$, $K = 1.5\times10^5\ M^{-1}$, $k_f = 1670\ s^{-1}$ and $k_b = 782\ s^{-1}$, an excellent fit has been obtained (see Fig. 3). The estimated error for K is $\pm6\times10^4\ M^{-1}$. In case of k_a value the error estimation procedure shows that k_a must be greater than $5\times10^8\ M^{-1}\cdot s^{-1}$ and any value greater than $10^9\ M^{-1}\cdot s^{-1}$ gives equally good fit.

4. DISCUSSION

The best-fit values of luminescence decay constants k_f and k_b are the same, within the error limits, as those measured for free terbium in buffer solution ($1670\ s^{-1}$) and for protein-coordinated terbium ($770\ s^{-1}$) [3,8], respectively. As expected the dissociation rate constant $k_d = k_a/K \approx 10^4\ s^{-1}$ is much greater than k_b, so that dissociation prior to quantum emission is a very probable event. The high value of k_a indicates that association is probably diffusion controlled.

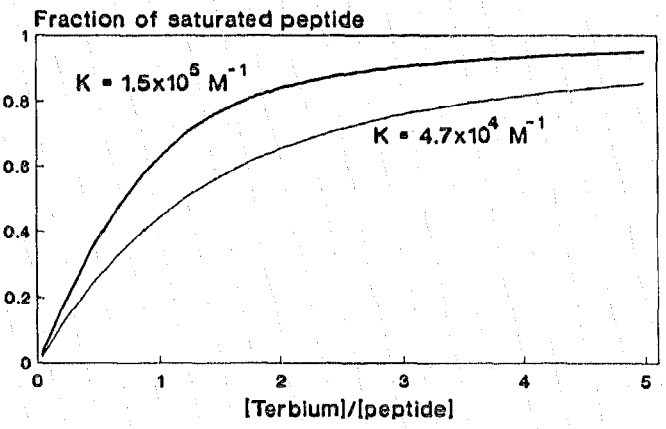

Fig. 4. Fraction of peptide molecules saturated by terbium calculated at peptide concentration of 30 μM using the binding constants determined by steady-state luminescence measurements ($K = 4.7\times10^4\ M^{-1}$, see text) and by terbium luminescence decay analysis ($K = 1.5\times10^5\ M^{-1}$).

Let us define the limiting conditions for applicability of Eqn 1 as: $k_a[P_f] < 0.2\ k_f$ or $k_d < 0.2\ k_b$. The last relation is equivalent to $K > 5\ k_a/k_b$. It means that the systems for which the rate of terbium association is diffusion controlled ($k_a = 1\times10^9\ M^{-1}\cdot s^{-1}$) can be described by equation 1 only if $K > 10^7\ M^{-1}$ or if the concentration of terbium binding molecules does not exceed 0.2 μM. In other cases determination of binding constants by simple measurements of terbium luminescence intensity are prone to serious errors. In our case the K value determined in this way was nearly one order of magnitude smaller than the correct one. In typical experiments peptide concentrations are kept at about 30 μM. Fig. 4 shows how large at this concentrations can be the errors made in calculation of a fraction of terbium saturated peptide molecules when the binding constant is determined from simple steady-state luminescence measurements.

It can be expected that all short protein fragments (40 amino acid residues or less) containing only one calcium binding loop are characterized by terbium binding constants much smaller than $10^7\ M^{-1}$ and by diffusion controlled binding rates. For many proteins the condition $K > 10^7\ M^{-1}$ may also not be fulfilled. But in proteins, the rate-limiting step of ion binding is usually related with conformational transition of a rigid protein molecule. Consequently, k_a can be reduced by orders of magnitude and eqn 1 can be valid, even if terbium binding constants are fairly low. Nevertheless, in each particular case, it should be checked out by luminescence decay measurements.

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