Binding of Lanthanide Ions to Troponin C†

Chih-Lueh A. Wang, Paul C. Leavis, W. DeW. Horrocks, Jr., and J. Gergely*

ABSTRACT: Tb^{3+} and Eu^{3+} bound to troponin C were detected by (1) changes in the fluorescence of the tyrosine chromophore of the protein or (2) the luminescence of the ions themselves excited by energy transfer from the protein or by direct excitation using a pulsed laser light source [Horrocks, W. DeW., Jr., & Sudnick, D. R. (1979) *Science (Washington, D.C.) 206*, 1194–1196]. Titrations carried out in the absence and presence of Ca^{2+} suggest two classes of binding sites (two sites in each class) for the lanthanides, corresponding to the high- and the low-affinity sites for Ca^{2+}. Computer analysis, assuming competition between Ca^{2+} and the lanthanide, using the binding constants of Ca^{2+} for TnC [Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628–4633] yields 5.2×10^8 M^{-1} and 9.7×10^6 M^{-1} for Tb^{3+} and 4.7×10^9 M^{-1} and 5.3×10^7 M^{-1} for Eu^{3+}, for the high- and the low-affinity sites, respectively. From lifetimes of laser-induced luminescence in H_2O and in D_2O, the number of water molecules coordinated to Eu^{3+} was two at the high and three at the low affinity sites.

Troponin C (TnC),[1] the Ca^{2+}-binding subunit of the troponin complex from mammalian skeletal muscle, contains four Ca^{2+}-binding sites (numbered from I to IV from the N terminus), each of which comprises ten amino acid residues, with at least four containing carboxylate groups that serve as Ca^{2+}-coordinating ligands (Collins et al., 1973). Two of these sites, sites III and IV, bind Ca^{2+} with a higher affinity ($K_1^{Ca} = 2 \times 10^7$ M^{-1}) and also bind Mg^{2+} (the Ca^{2+}–Mg^{2+} sites), and sites I and II apparently only bind Ca^{2+} (the Ca^{2+}-specific sites) with a lower affinity ($K_2^{Ca} = 5 \times 10^5$ M^{-1}) (Potter & Gergely, 1975).

In recent years, trivalent lanthanide ions (Ln^{3+}), which have been used in many systems as substitutes for Ca^{2+}, have been useful as reporter groups in the study of TnC and other Ca^{2+}-binding proteins, because they have in common with Ca^{2+} an ionic radius of about 1 Å, coordination numbers between 6 and 8, and a preference for coordination with oxygen rather than nitrogen atoms (Prados et al., 1974). In addition, several of these trivalent ions are paramagnetic and/or luminescent, and the luminescence may increase when the ion is bound to a protein close to an aromatic group (Luk, 1971). In the case of TnC, the quantum yield of the luminescent lanthanide, Tb^{3+}, extremely low for the free ion (excited at 280 nm), increases upon binding to the protein; Tb^{3+} serves as an acceptor of fluorescence energy transfer from Tyr-109, located in binding site III of TnC (Donato & Martin, 1974).

In a recent paper from this laboratory (Leavis et al., 1980), it has been shown that Tb^{3+} preferentially binds to the high-affinity sites of TnC, readily displacing Ca^{2+} and inducing structural changes in the protein similar to those induced by Ca^{2+}. In this work, we studied the binding of Tb^{3+} and Eu^{3+} to both classes of Ca^{2+}-binding sites on TnC by using two methods: (1) Ca^{2+} titration of TnC in the presence of Ln^{3+}, monitored by changes in the intrinsic fluorescence of Tyr-109 and tyrosine-excited Ln^{3+} luminescence, and (2) titration with direct excitation of the bound Eu^{3+} by using a pulsed dye laser. The second technique allows direct detection of Ln^{3+} bound to both classes of sites, which is not possible by energy transfer since no suitable donor fluorophore exists in the vicinity of the low-affinity sites. Finally, differences in the time course of the decay of the laser-induced luminescence of Eu^{3+} in H_2O and D_2O indicate that at the low-affinity sites there is one more water molecule in the coordination sphere of the metal ion.

Experimental Procedures

Materials. TnC was prepared from rabbit skeletal muscle as previously described (Potter & Gergely, 1974). The protein was rendered calcium free by addition of 5 mM EDTA followed by extensive dialysis. TnC solutions were checked for calcium contamination by addition of 1 mM EDTA and monitoring tyrosine fluorescence (cf. Kawasaki & van Eerd, 1972). No changes were observed; this indicates that contaminating Ca^{2+}, if any, was so low that there was no significant binding. $TbCl_3$ and $EuCl_3$, both in hexahydrated form (99.9%), were purchased from Alfa Products, Ventron Corp. D_2O, 99.7% pure, was obtained from Merck Isotopes. All other chemicals were reagent grade.

Methods and Data Analyses. Fluorescence measurements were recorded with a Hitachi Perkin-Elmer MPF-4 spectrofluorimeter equipped with a thermostated cell holder. Titrations based on Ca^{2+} competition were performed at 25 °C as follows. To TnC solutions containing various amounts of $TbCl_3$ or $EuCl_3$, was added $CaCl_2$ in small increments from either a Metrohm micriburet or a Hamilton syringe. During the titration, the fluorescence attributable to tyrosine (λ_{exc} = 280 nm; λ_{em} = 300 nm) increased upon Ca^{2+} binding, while the metal luminescence (λ_{exc} = 280 nm and λ_{em} = 543 or 490 nm for Tb^{3+}; λ_{exc} = 394 nm and λ_{em} = 615 nm for Eu^{3+}) decreased. These changes (in either tyrosine or metal luminescence) were interpreted as being due to displacement by Ca^{2+} of the Ln^{3+} ions bound to the high-affinity sites (see Results). It was further assumed that both Ca^{2+} and Ln^{3+} bind to the sites in the low-affinity class without contributing to the spectral changes. [TnC_1^{Ln}], the concentration of the high-affinity sites to which Ln^{3+} is bound, was obtained from

† From the Department of Muscle Research, Boston Biomedical Research Institute, Departments of Neurology and Biochemistry, Harvard Medical School, and the Department of Neurology, Massachusetts General Hospital, Boston, Massachusetts 02114 (C.-L.A.W., P.C.L., and J.G.), and the Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania 16802 (W.DeW.H., Jr.). *Received September 19, 1980.* This work was supported by grants from the National Institutes of Health (HL5949 to J.G.; HL20464 to P.C.L.; and GM23599 to W.DeW.H., Jr.), the National Science Foundation (J.G.), and the Muscular Dystrophy Association of America (J.G. and P.C.L.). C.-L.A.W. was supported by a National Institutes of Health training grant and is a postdoctoral fellow of the Muscular Dystrophy Association. P.C.L. is an Established Investigator of the American Heart Association.

[1] Abbreviations used: TnC, troponin C; Ln^{3+}, lanthanide ions; EDTA, ethylenediaminetetracetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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the fluorescence measurement as

$$[TnC_1^{Ln}] = 2[TnC]\frac{F - F_\infty}{F_0 - F_\infty} \quad (1)$$

where F is the fluorescence at a particular added Ca^{2+} concentration, F_0 is the fluorescence in the Ca^{2+}-free (but Ln^{3+}-bound) state, and F_∞ in the Ca^{2+}-bound state. The concentrations of those TnC molecules in which sites in class i (i = 1 and 2 for the high- and low-affinity sites, respectively) are free or occupied by Ca^{2+} or Ln^{3+}, viz., TnC_i, TnC_i^{Ca}, and TnC_i^{Ln}, were calculated with a computer program by Perrin & Sayce (1967) that computes the concentrations of various complexes and free species as a function of the concentration of the added metals and ligands, given the appropriate binding constants. Experimental values of binding constants of Ca^{2+} to TnC were used ($K_1^{Ca} = 2 \times 10^7$ M^{-1} and $K_2^{Ca} = 5 \times 10^5$ M^{-1}) (Potter & Gergely, 1975), while the Ln^{3+} binding constants were varied to obtain the best least-squares fit between $[TnC_1^{Ln}]$ derived from fluroescence data (eq 1) and $[TnC_1^{Ln}]$ values calculated with the computer program.

Values of $[TnC_1^{Ln}]$ were calculated for various pairs of Ln^{3+}-binding constants at $[Ln]_0/[TnC]$ ratios varying from 2 to 6 for each added Ca^{2+} concentration. From these calculated and measured values of $[TnC_1^{Ln}]$ at various concentrations of added Ca^{2+}, $[Ca]_j$, the deviation D (eq 2) was

$$D = \sum_{j=1}^{m}([TnC_1^{Ln}]_{calcd,j} - [TnC_1^{Ln}]_{obsd,j})^2 \quad (2)$$

plotted as a function of K_i^{Ln} (i = 1, 2). The minimum of D was determined from the plot, and the correpsonding K_i^{Ln} values were reported as the "best-fit" constants.

Selective excitation of the individual, bound Eu^{3+} ions was carried out as described by Horrocks & Sudnick (1979b). The Eu^{3+}-containing samples were irradiated with a pulsed N_2 laser-pumped (10 Hz) dye laser as the excitation wavelength was scanned thorugh the 578–580-nm region. The emission monochromator was set with wide slits to accept the intense $^5D_0$–$^7F_2$ emission at 612 nm. The laser-excited emission as a function of excitation wavelength for various Eu^{3+} and Ca^{2+} concentrations was recorded and analyzed by least-squares fitting of p Lorentzian curves (p = 2 in this case; see Results), varying the maximal intensity (I_{max}), the wavelength at which I_{max} occurs (λ_0), and the half-width at $I_{max}/2$, $\Delta_{1/2}$, according to eq 3.

$$I(\lambda) = \sum_{i=1}^{p}\frac{I_{max,i}}{1 + \left[\frac{\lambda - \lambda_{0,i}}{\Delta_{1/2,i}}\right]^2} \quad (3)$$

For measurements of the luminescence decay constants, experiments were performed as previously described (Horrocks & Sudnick, 1979a).

Results

Titration of apo-TnC with Tb^{3+}, monitored by the luminescence of the metal, shows an apparent stoichiometry of 2:1 (Figure 1), as reported previously. In contrast to the titration of TnC with Ca^{2+}, titration with Tb^{3+} does not produce an enhancement of the tyrosine fluorescence but rather produces a decrease of about 10%. If a Tb^{3+} titration is carried out on a 2:1 $[Ca]_0/[TnC]$ complex, the increase in Tb^{3+} luminescence is accompanied by a large decrease in the tyrosine fluorescence, resulting from the displacement of Ca^{2+} by the lanthanide ion. It should be noted that, although optical studies do not detect binding of Ln^{3+} to the low affinity sites, this should not be taken as evidence for lack of binding to the latter. In fact, as shown below, Ca^{2+} titration studies indeed suggest such binding.

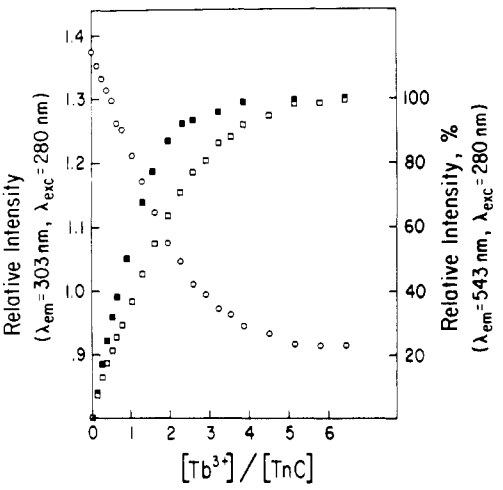

FIGURE 1: Terbium titration of apo-TnC and Ca_2TnC. (□, ■) Enhanced Tb^{3+} luminescence (λ_{ex} 280 nm and λ_{em} 543 nm); (○) protein intrinsic fluorescence expressed as the ratio of measured fluorescence and that of the apo-TnC (λ_{ex} 280 nm and λ_{em} 303 nm); (○, □) Ca_2TnC; (■) apo-TnC. Portein concentrations are 20 μM in 25 mM Pipes buffer containing 0.1 M KCl, pH 6.9.

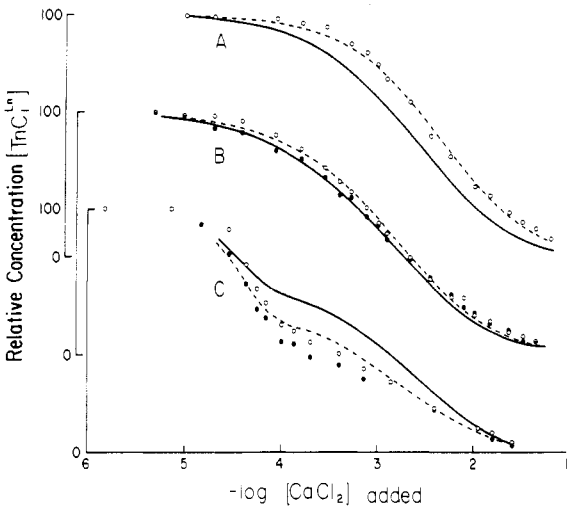

FIGURE 2: Competitiion between Ca^{2+} and Tb^{3+} for TnC. Added Tb^{3+}/TnC is 6 (A), 4 (B), and 2 (C). (○, ●) Experimental points based on the spectral changes of metal luminescence and tyrosine fluorescence, respectively. Dotted lines were calculated with binding constants that best fit each individual titration. Solid curves were calculated with the averaged best-fit constants (see Experimental Procedures).

For determination of the binding affintiy of Tb^{3+} to both classes of sites on TnC, $CaCl_2$ was added to solutions containing Tb^{3+} and TnC at various ratios, n (n ranges from 2 to 6). As more Ca^{2+} was added, Tb^{3+} emission decreased while tyrosine emission increased, reflecting Ca^{2+} displacement of Tb^{3+} from the protein. The titrations (Figure 2) extended over at least 3 orders of magnitude of added Ca^{2+} at all $[Tb]_0/[TnC]$ ratios. While there is considerable difference between the shapes of the Ca^{2+} titration curves with n = 2 and n = 4, the shape of the curve for n = 6 differs very little from that with n = 4. Some curves calculated according to the procedures described under Experimental Procedures with the constants in the vicinity of the minimum of the $D(K_1^{Ln}, K_2^{Ln})$ surface (Figure 3), are also shown in Figure 2.

These curve fittings (as described in the above section) were based on a model with two independent classes of metal binding sites, each class having two equivalent sites. Poor fits

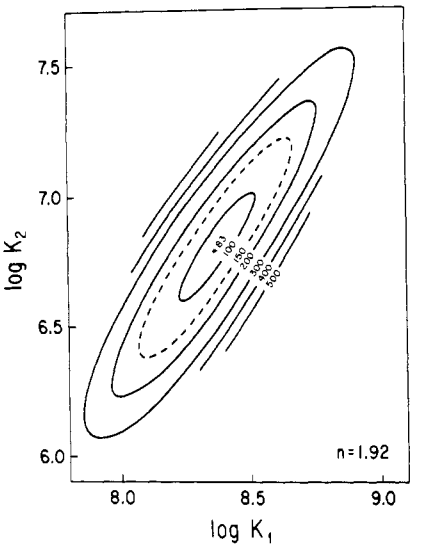

FIGURE 3: The *D*-surface diagram. The deviation *D* (in arbitrary unit) wa s plotted as a function of both K_1^{Ln} and K_2^{Ln}. In the case shown, Ln = Tb and n = 1.92 (see Experimental Procedures).

Table I: Summary of K_i^{Ln} Values[a]

Ln^{3+}	n	emitting groups	$10^{-8}K_1^{Ln}$ (M^{-1})	$10^{-7}K_2^{Ln}$ (M^{-1})
Tb^{3+}	2.0	metal ions	2.4	0.63
		tyrosine	2.7	1.19
	3.0	metal ions	4.2	0.71
		tyrosine	4.7	1.26
	4.0	metal ions	5.9	0.32
		tyrosine	7.2	1.70
	6.0	metal ions	9.3	
Eu^{3+}	2.0	tyrosine	25.0	0.80
	2.1	metal ions	50.0	12.6
		tyrosine	20.0	4.5
	4.0	tyrosine	94.0	3.2

[a] Averaged: for Tb^{3+}, $K_1 = (5.2 \pm 2.5) \times 10^8$ M^{-1} and $K_2 = (9.7 \pm 5.0) \times 10^6$ M^{-1}; for Eu^{3+}, $K_1 = (4.7 \pm 3.4) \times 10^9$ M^{-1} and $K_2 = (5.3 \pm 5.1) \times 10^7$ M^{-1}.

were obtained if a single class of sites was assumed; for a single class of binding sites, the transition would be much steeper. Data were also analyzed by using three classes of sites (total of six sites), but no better fit was obtained, and the binding constants associated with the postulated, third class of sites were less than 10^3 M^{-1} for Tb^{3+}, indicating that these sites, if they exist, bind metal ions (Ln^{3+}) only very weakly. Table I lists the results of the curve fitting and the averaged best-fit values of binding constants.

In another approach, the Ln^{3+}–TnC binding system was examined by direct excitation with a pulsed dye laser. Eu^{3+} was chosen for this purpose because of its nondegenerate ground ($^7F_0$) and excited ($^5D_0$) states in either the free or the bound form; the $^7F_0 \rightarrow {}^5D_0$ transition corresponds to excitation in the 578–580-nm region. For a single complex of Eu^{3+} in solution, a single sharp transition would be expected. In the TnC binding system, however, two peaks were detected, indicating at least two classes of complexes (Figure 4). The first peak to appear as increasing amounts of Eu^{3+} are added is at λ_{max} 578.9 nm (peak I). We therefore identify it with the binding of Eu^{3+} to the high-affinity sites. The second, more intense peak (peak II) at λ_{max} 579.2 nm corresponds to binding to the sites of lower affinity.

The λ_{max} values of the two peaks do not change as the Ln^{3+}/TnC ratio is varied. The I_{max} values are not consistent with the occupancy of the sites calculated with the constants in Table I. Presumably, the poor resolution of the two peaks

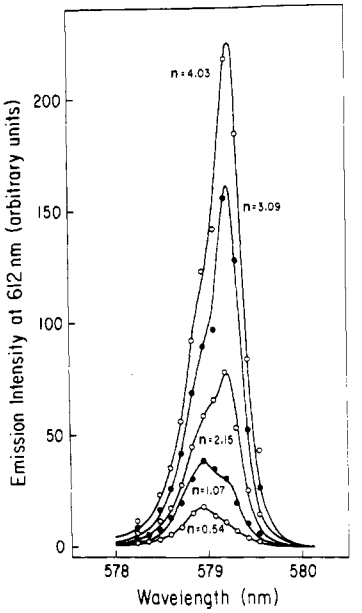

FIGURE 4: Typical excitation spectra of laser-induced Eu^{3+} luminescence as apo-TnC is titrated with $EuCl_3$. λ_{em} 612 nm; [TnC] = 4.2×10^{-4} M; n stands for the ratio of added [Eu^{3+}] to [TnC]. Open and closed circles are best-fit values assuming two Lorentzian peaks. The I_{max} of peak II is larger than that of peak I, suggesting a higher quantum yield or molar excitation coefficient for the low-affinity sites than for the high-affinity sites.

at higher $[Ln]_0/[TnC]$ ratios introduces an error in the curve-fitting procedure. At present, therefore, the I_{max} values cannot be taken as quantitative measures of the occupancy of the two classes of sites.

Addition of Ca^{2+} in the presence of 1 mol of Eu^{3+} per mol of TnC causes a decrease in peak I and an increase in peak II. This indicates that as Ca^{2+} binds to the protein, Eu^{3+} originally bound to the high-affinity sites goes to the low-affinity sites. When 4 mol of Eu^{3+} per mol of TnC is present, the addition of Ca^{2+} decreases the intensity of both peaks, which is consistent with Eu^{3+} ions at both high- and low-affinity sites being displaced (Table II).

Reciprocal lifetimes, τ^{-1}, for the $^5D_0$ state of Eu^{3+} for excitation of both peak I and peak II were determined in H_2O as well as in D_2O. By applying the quantitative relationship (Horrocks & Sudnick, 1979b) between $\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1}$ and the number of coordinated water molecules (q) in the first coordination sphere of Eu^{3+} ion, the q values for both species corresponding to peaks I and II were estimated (Table III). The averaged q's are 2.1 ± 0.2 for peak I and 2.8 ± 0.5 for peak II, suggesting that there is one more water molecule coordinated to Eu^{3+} at the low-affinity sites than at the high-affinity sites.

Discussion

Although lanthanide ions have proven valuable with a large number of proteins as substitutes for Ca^{2+}, their use in TnC and its homologues is complicated by the multiplicity of binding sites and by the fact that these sites fall into two classes differing in their binding affinities. In a recent paper (Leavis et al., 1980), we showed that Tb^{3+} exhibited the same preferential binding to sites III and IV as does Ca^{2+} and induced comparable structural changes in the protein. However, owing to the lack of intrinsic fluorophores to serve as energy donors outside of the high-affinity sites region of the protein, we could not detect Tb^{3+} binding directly beyond a molar ratio of 2, nor could we determine numbers of sites and affinities for Tb^{3+} bound to the two classes of sites.

Table II: Calculated Parameters for Eu-TnC Complexation with Laser-Induced Luminescence

$[Eu^{3+}]/[TnC]^a$	$[Ca]_{tot}$ (mM)	λ_1 (nm)	$I_{max_1}{}^b$	$\Delta_{1/2,1}$ (nm)	λ_2 (nm)	$I_{max_2}{}^b$	$\Delta_{1/2,2}$ (nm)
0.54	0	578.92	17.6	0.21	579.24	5.2	
1.07	0	578.93	34.3	0.23	579.20	11.6	0.21
2.15	0	578.94	51.2	0.22	579.23	59.6	0.13
3.09	0	578.95	60.3	0.23	579.24	153.5	0.11
4.03	0	578.95	78.7	0.23	579.24	218.3	0.19
4.03	2.9	578.96	76.2	0.17	579.25	229.0	0.19
4.03	5.4	578.99	86.6	0.19	579.26	195.5	0.19
4.03	12.4	578.98	30.9	0.17	579.26	83.2	0.22
2.15	0	578.94	42.3	0.21	579.23	70.2	0.13
2.15	38	578.99	45.9	0.18	579.25	87.5	0.22
1.01	0	578.93	32.2	0.20	579.22	18.9	0.11
1.01	2.93	578.92	18.1	0.21	579.22	53.7	0.20
average		578.95 ± 0.02		0.20 ± 0.03	579.24 ± 0.02		0.17 ± 0.04

[a] Protein concentration [TnC] = 4.2×10^{-4} M. [b] Corrected for dilution effect.

Table III: Reciprocal Lifetimes of Eu^{3+} ($0 \longrightarrow 0$) Excitation

	peak I			peak II		
n	$\tau_{H_2O}{}^{-1}$ (ms^{-1})	$\tau_{D_2O}{}^{-1}$ (ms^{-1})	q	$\tau_{H_2O}{}^{-1}$ (ms^{-1})	$\tau_{D_2O}{}^{-1}$ (ms^{-1})	q
0.5	2.40	0.47	2.0	2.94	0.44	2.6
1.0	2.37	0.47	2.0	2.79	0.44	2.3
4.0	2.81	0.46	2.3	3.99	0.43	3.4

In the current report, we have employed two approaches to study binding parameters for lanthanide ions to TnC. In one case, Ca^{2+} titrations have been performed on TnC containing 2–6 mol of added Tb^{3+} or Eu^{3+} per mol of protein. The displacement of Ln^{3+} from the high-affinity sites was monitored either by recording the decrease in tyrosine-excited metal luminescence or by the increase in the fluorescence of Tyr-109 as Ca^{2+} occupies these sites. Binding of Ln^{3+} to the two low affinity sites, although not demonstrable by such titrations, can be inferred from the curve-fitting procedures which, as reported, give best fits by assuming two classes of two sites.

Direct demonstration of a second class of sites is obtained by the second approach, viz., examination of the excitation spectrum resulting from direct laser-induced luminescence of TnC-bound Eu^{3+}. Of particular interest is the exact position of the $^7F_0 \rightarrow {}^5D_0$ transition in the 580-nm range, since the wavelength of this transition is dependent on the local environment of the ion. In our system, two peaks emerge during sequential addition of Eu^{3+}, and the stoichiometries indicate that they correspond to the two classes of binding sites in TnC. This assignment is further supported by the fact that binding of Eu^{3+} to one of the proteolytic fragments of TnC (TR1E), which contains only the low-affinity sites (sites I and II), results in only one peak at the position of peak II (579.2 nm) (C.-L. A. Wang, unpublished results). The presence of only two peaks for TnC suggests that the two sites within each class possess essentially identical chemical environments with respect to the bound ion. As indicated above, the heights of the two peaks cannot be taken as quantities of the fraction of each class of sites occupied.

From the measurements made by indirect excitation, the affinities of Ln^{3+} ions for the metal binding sites of TnC were estimated. With these binding constants on hand, one can calculate the distribution of lanthanide ions among the four sites at any Ca^{2+} concentration. Figure 5 shows such a calculated result generated by the computer program for $n = 1$, 2, 3, and 4 with Tb^{3+} and $n = 2$ with Eu^{3+}. It can be seen from these plots that the lanthanide ions originally bound to the high-affinity sites are displaced to available low-affinity sites (i.e., $n < 4$) by added Ca^{2+} before they are eventually displaced from the protein (for details, see Appendix).

The binding constants of Ln^{3+} toward a given Ca^{2+} binding site are greater than the corresponding value for Ca^{2+} (Table I). In the case of parvalbumin, paramagnetic resonance studies (Cave et al., 1979) show that the affinity of Ln^{3+} for the third site is 2 orders greater than that of Ca^{2+}. Strong interactions between macromolecules and Ln^{3+} are also found for many other systems [for a review, see Martin & Richardson (1979)]. The practical consequence of this is that the complete substitution of Ca^{2+} with Ln^{3+} can be easily achieved. It should be pointed out that, although averages of the constants obtained at different $[Tb]_0/[TnC]$ and $[Eu]_0/[TnC]$ ratios are given for purposes of comparison with the Ca^{2+} binding constants, there seems to be a trend for the constants to increase with the Ln^{3+}/TnC ratio. This may indicate a weak positive cooperativity; more work would be required for full statistical evaluation.

While the charge effect and the ionic size effect have been used to account for Ln^{2+} having a higher affinity for TnC than Ca^{2+}, there is another thermodynamic consideration that should be discussed. Complex formation between Ln^{3+} and many proteins is found to be an endothermic process (Epstein et al., 1977), suggesting that the higher binding constants or more negative free energies of binding are the result of a large positive entropy change. Positive entropy changes can be attributed to the extensive dehydration of the cation accompanying complex formation (Choppin, 1971). It is known that the hydration numbers, q, for the aquo ions are 9 or 10 for Eu^{3+} and 6 for Ca^{2+} (Williams, 1976; Cummings et al., 1980). By analogy with the third site of parvalbumin (Moews & Kretsinger, 1975), we estimate that there is a single water molecule at the high-affinity sites on TnC when occupied by Ca^{2+}. Thus, five water molecules are lost by each Ca^{2+} ion upon binding to such sites. Our lifetime measurements indicate that there are two H_2O molecules in the first coordination sphere of Eu^{3+} bound to the high-affinity sites, suggesting a loss of about seven or eight water molecules upon dehydration. Assuming that the entropy of the complexation with proteins is due solely to dehydration, one concludes that the higher binding constants of Eu^{3+} can be fully accounted for by this extensive dehydration upon binding to the protein. A similar argument can be applied when the binding parameters of Eu^{3+} and Tb^{3+} are compared. Since $q = 9$ for aquo-Tb^{3+} (Horrocks & Sudnick, 1979a) and, presumably, $q = 2$ at the high-affinity sites, the same as for Eu^{3+}, seven water molecules are lost. The dehydration of Tb^{3+} is not as extensive as that of Eu^{3+}, corresponding to a slight difference in the binding affinity. Moreover, our data indicate that there are three H_2O coor-

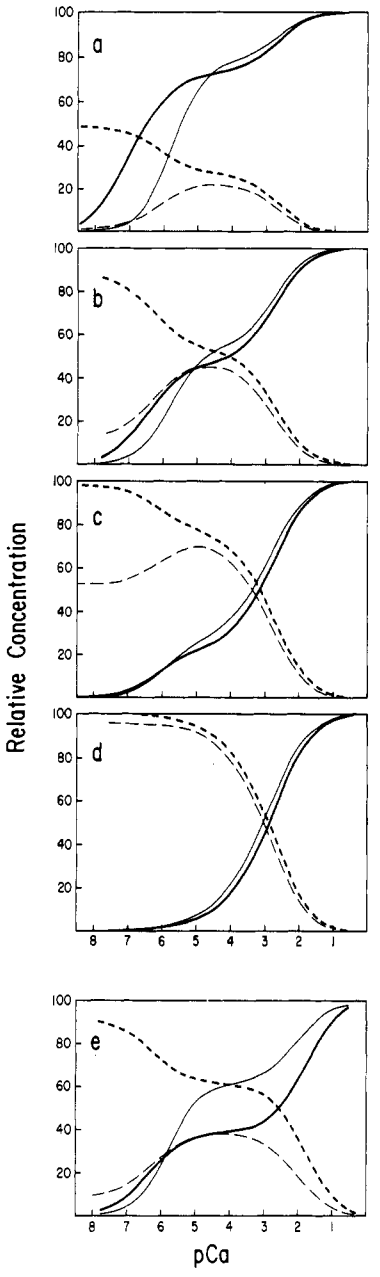

FIGURE 5: Calculated relative concentrations of metal ions as a function of free Ca^{2+} concentration with $n = 1, 2, 3$, and 4 for Tb^{3+} and $n = 2$ for Eu^{3+} with the use of the averaged binding constants for Ln^{3+} in Table I. (Heavy solid lines) Ca^{2+} in high-affinity sites; (heavy dashed lines) Ln^{3+} in high-affinity sites; (light solid lines) Ca^{2+} in low-affinity sites; (light dashed lines) Ln^{3+} in low-affinity sites. (a) Tb^{3+}, $n = 1$; (b) Tb^{3+}, $n = 2$; (3) Tb^{3+}, $n = 3$; (d) Tb^{3+}, $n = 4$; (e) Eu^{3+}, $n = 2$.

dinated to Eu^{3+} at the low-affinity sites, again consistent with the observed lower affinities in sites I and II.

Acknowledgments

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Appendix

The distribution of Ca^{2+} A2 Ln^{3+} between the two classes of binding sites, P^1 and P^2, during a competition titration can be calculated as

$$K_1^{Ca}[P^1][Ca] = [P^1\text{–}Ca] \quad \text{(A1)}$$

$$K_2^{Ca}[P^2][Ca] = [P^2\text{–}Ca] \quad \text{(A2)}$$

where K_1^{Ca} and K_2^{Ca} are the association constants for Ca^{2+} to the two classes of sites, P^1 and P^2 are the free sites, and P^1–Ca and P^2–Ca are the occupied sites. From eq A1 and A2

$$\frac{K_1^{Ca}[P^1]}{K_2^{Ca}[P^2]} = \frac{[P^1\text{–}Ca]}{[P^2\text{–}Ca]} \quad \text{(A3)}$$

Similarly for Ln^{3+}

$$\frac{K_1^{Ln}[P^1]}{K_2^{Ln}[P^2]} = \frac{[P^1\text{–}Ln]}{[P^2\text{–}Ln]} \quad \text{(A4)}$$

From eq A3 and A4

$$\frac{[P^1\text{–}Ca]}{[P^2\text{–}Ca]} = \frac{K_1^{Ca}K_2^{Ln}}{K_2^{Ca}K_1^{Ln}}\frac{[P^1\text{–}Ln]}{[P^2\text{–}Ln]} \quad \text{(A5)}$$

Let $[P^2\text{–}Ca]/[P^1\text{–}Ca] = r_{Ca}$, $[P^2\text{–}Ln]/[P^1\text{–}Ln] = r_{Ln}$, and $(K_1^{Ln}K_2^{Ca})/(K_1^{Ca}K_2^{Ln}) = K$. Then eq A5 becomes

$$r_{Ca} = Kr_{Ln} \quad \text{(A6)}$$

Let f_1 and f_2 be the fractions of empty sites for class 1 and 2, respectively.

$$f_1 = \frac{[P^1]}{[P^1]_0} = \frac{[P^1]}{[P^1] + [P^1\text{–}Ca] + [P^1\text{–}Ln]} = \frac{1}{1 + K_1^{Ca}[Ca] + K_1^{Ln}[Ln]} \quad \text{(A7)}$$

and

$$f_2 = \frac{1}{1 + K_2^{Ca}[Ca] + K_2^{Ln}[Ln]} \quad \text{(A8)}$$

From (A3) and the fact that $[P^1]_0 = [P^2]_0$

$$r_{Ca} = \frac{[P^2\text{–}Ca]}{[P^1\text{–}Ca]} = \frac{K_2^{Ca}[P^2]}{K_1^{Ca}[P^1]} = \frac{K_2^{Ca}f_2}{K_1^{Ca}f_1} = \frac{K_2^{Ca}(1 + K_1^{Ca}[Ca] + K_1^{Ln}[Ln])}{K_1^{Ca}(1 + K_2^{Ca}[Ca] + K_2^{Ln}[Ln])} \quad \text{(A9)}$$

According to (A9), (i) when [Ca] is very small (in the initial stage of titration)

$$r_{Ca} = \frac{K_2^{Ca}(1 + K_1^{Ln}[Ln])}{K_1^{Ca}(1 + K_2^{Ln}[Ln])} \quad \text{(A10)}$$

(a) If [Ln] is also small, so that $1 \gg K_i^{Ln}[Ln]$ ($i = 1, 2$), then

$$r_{Ca} = K_2^{Ca}/K_1^{Ca} \quad \text{(A11)}$$

Thus the distribution of Ca^{2+} between the two classes depends only on the affinity constants for Ca^{2+}. In actuality, this is the case as long as the sites are not saturated with lanthanide ions.

(b) If [Ln] is large, so that $K_i^{Ln}[Ln] \gg 1$, then

$$r_{Ca} = \frac{K_2^{Ca}K_1^{Ln}}{K_1^{Ca}K_2^{Ln}} = K \quad \text{(A12)}$$

and, according to (A6), $r_{Ln} = 1$.

This is the case when both high-affinity and low-affinity sites are saturated with Ln^{3+}. The distribution of Ca^{2+} will depend on all affinities. It should be noted that in general the high-affinity sites for Ca^{2+} can be the low-affinity sites of Ln^{3+}, and vice versa.

(ii) When [Ca] is very large (at the end of titration), from

(A9), $r_{Ca} = 1$; both classes of sites are saturated with Ca^{2+} to the same extent. At this stage, according to (A6), $r_{Ln} = 1/K$, and it is the distribution of the residual bound Ln^{3+} that depends on all the affinities.

For nonsaturating [Ln], the added Ca^{2+} will, according to (i)(a), bind preferentially to the high-affinity sites; this first causes redistribution of Ln^{3+} from the high- to the low-affinity sites, resulting in a temporary accumulation of Ln^{3+} at the low-affinity sites. As [Ca] increases, Ln^{3+} is eventually displaced from the protein into solution.

References

Cave, A., Daures, M.-F., Parello, J., Saint-Yves, A., & Sempere, R. (1979) *Biochimie 61*, 755.

Choppin, G. R. (1971) *Pure Appl. Chem. 27*, 23.

Collins, J. H., Potter, J. D., Horn, M. J., Wiltshire, G., & Jackman, N. (1973) *FEBS Lett. 36*, 268.

Cummings, S., Enderby, J. E., & Howe, R. A (1980) *J. Phys. C 13*, 1.

Donato, H., Jr., & Martin, R. B. (1974) *Biochemistry 13*, 4575.

Epstein, M., Reuben, J., & Levitzki, A. (1977) *Biochemistry 16*, 2449.

Horrocks, W. DeW., Jr., & Sudnick, D. R. (1979a) *J. Am. Chem. Soc. 101*, 334.

Horrocks, W. DeW., Jr., & Sudnick, D. R. (1979b) *Science (Washington, D.C.) 206*, 1194.

Kawasaki, Y., & van Eerd, J. P. (1972) *Biochem. Biophys. Res. Commun. 49*, 898.

Leavis, P. C., Nagy, B., Lehrer, S. S., Bialkowska, H., & Gergely, J. (1980) *Arch. Biochem. Biophys. 200*, 17.

Luk, C. K. (1971) *Biochemistry 10*, 2838.

Martin, R. B., & Richardson, F. S. (1979) *Q. Rev. Biophys. 12*, 181.

Moews, P. C., & Kretsinger, R. H. (1975) *J. Mol. Biol. 91*, 229.

Perrin, D. D., & Sayce, I. G. (1967) *Talanta 14*, 833.

Potter, J. D., & Gergely, J. (1974) *Biochemistry 13*, 2697.

Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628.

Prados, R., Stadtherr, L. G., Donato, H., Jr., & Martin, R. B. (1974) *J. Inorg. Nucl. Chem. 36*, 689.

Williams, R. J. P. (1976) *Proc. Soc. Exp. Biol. 30*, 1.

Structural Identification of Autoinducer of *Photobacterium fischeri* Luciferase†

A. Eberhard,* A. L. Burlingame, C. Eberhard,‡ G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer

ABSTRACT: Synthesis of bacterial luciferase in some strains of luminous bacteria requires a threshold concentration of an autoinducer synthesized by the bacteria and excreted into the medium. Autoinducer excreted by *Photobacterium fischeri* strain MJ-1 was isolated from the cell-free medium by extraction with ethyl acetate, evaporation of solvent, workup with ethanol–water mixtures, and silica gel chromatography, followed by normal-phase and then reverse-phase high-performance liquid chromatography. The final product was >99% pure. The structure of the autoinducer as determined by high-resolution ^1H nuclear magnetic resonance spectroscopy, infrared spectroscopy, and high-resolution mass spectrometry was *N*-(3-oxohexanoyl)-3-aminodihydro-2(3*H*)-furanone [or *N*-(β-ketocaproyl)homoserine lactone]. The formation of homoserine by hydrolysis of the autoinducer was consistent with this structure. Synthetic autoinducer, obtained as a racemate, was prepared by coupling homoserine lactone to the ethylene glycol ketal of sodium 3-oxohexanoate, followed by mildly acidic removal of the protecting group; this synthetic material showed the appropriate biological activity.

Bacterial luciferase, a mixed function oxidase, catalyzes the reaction

$$O_2 + RCHO + FMNH_2 \xrightarrow{\text{luciferase}} RCOOH + FMN + H_2O + h\nu$$

Both its synthesis and level of activity are regulated by a highly complex set of control mechanisms (Hastings & Nealson, 1977; Nealson & Hastings, 1979).

The synthesis of luciferase in *Photobacterium fischeri* can be turned on and off by the bacteria themselves in a unique way. Bacteria excrete an autoinducer into the medium that allows induction of luciferase synthesis when its concentration reaches a critical level (Nealson et al., 1970; Eberhard, 1972; Nealson, 1977). The *P. fischeri* autoinducer is species specific, inducing only other strains of *P. fischeri* and no other luminous species (Eberhard, 1972; Nealson, 1977). In addition, complex nutrient media contain an inhibitor of luciferase synthesis, so that when bacteria are inoculated into fresh medium, luciferase synthesis is temporarily repressed. The bacteria must remove the inhibitor metabolically in addition to excreting a sufficient amount of autoinducer before luciferase synthesis can begin (Kempner & Hanson, 1968; Eberhard, 1972). The inhibitor is not species specific (Eberhard, 1972) and can be removed by different bacterial species. Luciferase synthesis must thus be indirectly controlled by the levels of enzymes involved in the metabolism of the inhibitor and synthesis of the autoinducer.

In *P. fischeri* and other luminous species several additional factors can also affect luminescence. These include the types and levels of carbohydrates in the growth medium, oxygen tension, cyclic nucleotides, temperature, salt concentration,

† From the Department of Chemistry, Ithaca College, Ithaca, New York 14850 (A.E.), the Department of Marine Biology, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093 (K.H.N.), and the Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143 (A.L.B., G.L.K., and N.J.O.). *Received September 30, 1980.* This work was supported in part by U.S. Public Health Service Grants AM 17323 (G.L.K.) and GM 22982 (N.J.O) and by a grant from the National Science Foundation (K.H.N.). Mass spectrometry was supported by National Institutes of Health Grant RR 00719 (A.L.B.), Division of Research Resources. G.L.K. is a National Institutes of Health Research Career Development Awardee (AM 00014, 1975-1980). N.J.O. is also a National Institutes of Health Research Career Development Awardee (CA 00587, 1979–1984). A preliminary report of this work was presented at the 80th Annual Meeting of the American Society for Microbiology, Miami Beach, FL, May 1980 (Eberhard et al., 1980).

‡ On leave from the Division of Biological Sciences, Cornell University, Ithaca, NY 14853.

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