The Distance between the High Affinity Sites of Troponin-C Measured by Interlanthanide Ion Energy Transfer*

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Trivalent lanthanide ions are known to be good substituents for Ca2+ at all four calcium-binding sites of rabbit skeletal troponin-C (TnC). In particular, the visible luminescence of terbium ions bound to the two high affinity sites can be excited via a tyrosine residue. We have carried out energy transfer measurements using Tb3+ as the donor and a number of lanthanide ions as acceptors in order to measure the distance between the two high affinity sites in TnC. Luminescence decay measurements showed that, in the absence of acceptors, TnC-bound Tb3+ decays with a single lifetime of 1.31 ms. In the presence of a good acceptor such as Nd3+, the decay was resolved into two components of roughly equal amplitude. The first was the same in lifetime as that of TnC-bound Tb3+ alone; the second has a shorter lifetime, presumably due to interlanthanide ion energy transfer. From these lifetimes and published critical transfer distances (Horrocks, W. DeW., Jr., Rhee, M.-J., Snyder, A. P., and Sudnick, D. R. (1980) J. Am. Chem. Soc. 102, 3650-3652), we obtained a distance of 0.92 nm between the two high affinity sites. This distance is consistent with the fact that the two high affinity sites have been located in the COOH-terminal half of TnC and with the proposal that the two halves of TnC is homologous in structure to parvalbumin (Kretsinger, R. H., and Barry, C. D. (1975) Biochim. Biophys. Acta 405, 40-52).

In recent years, the use of the singlet-singlet energy transfer technique has become increasingly popular for mapping distances between specific sites in macromolecular systems (Stryer, 1978; Fairclough and Cantor, 1978). Most of these measurements suffer from a major disadvantage. It is impossible to experimentally determine the value of the orientation factor $x^2$ unambiguously. Although methods exist to limit the range of $x^2$ (Dale and Eisinger, 1975), such distance measurements are, nevertheless, subject to a certain degree of uncertainty. Energy transfer between metal ions has been observed (Horrocks et al., 1975; Berner et al., 1975), and more recently, distance determinations have been carried out successfully using metal ions as donor-acceptor pairs (Rhee et al., 1981; Snyder et al., 1981; O'Hara et al., 1981). Owing to multiple degeneracies in the electronic levels, the transition moments of the metal ions have no discrete orientation, and $x^2$ averages to exactly two-thirds (Horrocks et al., 1975). Metal-metal energy transfer measurements, therefore, provide a means to unambiguously measure distances between metal-binding sites in macromolecules.

The present studies were carried out to measure distances by means of metal-metal energy transfer between calcium-binding sites in troponin-C, the calcium-binding subunit of the regulatory muscle protein troponin. The contraction of mammalian skeletal muscle is triggered when calcium ions released from the sarcoplasmic reticulum are bound to TnC.

TnC contains four calcium-binding sites (Potter and Gergely, 1975), of which two (I and II) identified as the low affinity sites are in the NH2-terminal half of the polypeptide chain, while the two (III and VI) high affinity sites are in the COOH-terminal half (Leavis et al., 1978). A number of trivalent lanthanide ions can substitute for calcium ions at all four binding sites (Wang et al., 1981). In particular, when Tb3+ is added to TnC at a stoichiometry of 2:1 or less, it binds only to the two high affinity sites (Leavis et al., 1980). Furthermore, the luminescence of TnC-bound Tb3+ is enhanced when excited with ultraviolet light of wavelength ~280 nm, evidently due to energy transfer from a nearby tyrosine residue (Tyr-109) (Donato and Martin, 1974). We have observed energy transfer from Tb3+ to a number of lanthanide ion (Ln3+) acceptors by indirect excitation via Tyr-109 and measuring the luminescence decay of complexes composed of Tb3+, Ln3+, and TnC at a molar ratio of 1:1:1. Energy transfer efficiencies were obtained from the decrease in the lifetime of the Tb3+ luminescence. Using published critical transfer distances, a value of 0.92 nm was obtained for the distance between the two high affinity sites of TnC. This distance is comparable with the distance between the calcium-binding sites in parvalbumin.

**THEORY**

The theory of Förster energy transfer is well established (Stryer, 1978; Fairclough and Cantor, 1978). Briefly, the efficiency of energy transfer, $E$, is given by

$$E = 1 - \frac{\tau_d}{\tau_d + \tau_a} = \frac{1}{1 + (r/R_0)^6}$$

where $\tau_d$ and $\tau_a$ are the luminescence lifetimes of the donor in the presence and absence of acceptor, respectively, $r$ is the actual donor-acceptor separation, and $R_0$, the critical distance for 50% energy transfer is given by

$$R_0 = (8.78 \times 10^{-8}) x^2 \phi n^{-1/3} \text{ cm}^6$$

where $x^2$ is the orientation factor, $\phi$ is the quantum yield of Tb3+.

*The abbreviations used are: TnC, troponin-C; Tb-TnC, sample composed of Tb3+ and TnC at a molar ratio of 1:1; Tb3+-TnC, sample composed of Tb3+ and TnC at a molar ratio of 2:1; Tb-Ln-TnC, sample composed of Tb3+, Ln3+ (a lanthanide ion), and TnC at a molar ratio of 1:1:1; e.g., Tb-Nd-TnC is a sample composed of Tb3+, Nd3+, and TnC at a molar ratio of 1:1:1. TnC, trypsin digest fragment of TnC containing residues numbered 89-159.

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the donor in the absence of the acceptor, \( n \) is the refractive index of the intervening medium between the donor and the acceptor, and \( J \) is the overlap integral of the donor emission spectrum and the acceptor absorption spectrum.

Using Equation 2, Horrocks et al. (1980) had determined \( R_0 \) for a number of lanthanide ion donor-acceptor pairs. In their determinations, \( J \) was obtained by numerical integration, \( \phi \) was taken to be 0.49, \( n \) was taken to be 1.35, and \( \kappa^2 \) was taken to be two-thirds on the basis that the presence of multiple degeneracies in lanthanide ions randomizes the relative donor-acceptor transition moment orientations. Using these published values of \( R_0 \) and experimentally determined values of \( \tau_{d0} \) and \( \tau_{a0} \), we could obtain \( r \) from Equation 1 readily.

**Materials and Methods**

TnC was prepared from rabbit skeletal muscle as described (Potter and Gergely, 1974). TmC was prepared by tryptic digestion of TnC (Drabikowski et al., 1977). Lanthanide ions (99.9% purity) were purchased from Alpha Ventron (Danvers, MA) in their hexahydrated chloride forms. All other reagents were obtained from commercial sources at reagent grade.

\( \text{Tb}^{3+} \) luminescence decay measurements were carried out on a laboratory-built apparatus; a block diagram of the apparatus is shown in Fig. 1. Since the luminescence can be excited through a tyrosine residue, a medium intensity ultraviolet flash lamp sufficed as the excitation source. The excitation filter was so chosen that only protein aromatic residues were excited. A photomultiplier equipped with a gating circuit was used as the detector. A triggering circuit was used to trigger the flash lamp and the signal averager. Simultaneously, the photomultiplier is gated off for 0.1 ms in order to prevent overloading the tube with scattered light or prompt luminescence. Decay data from 200-300 sweeps were signal averaged in 256 channels. The digital data points were transferred serially to a PDP 11/03 microcomputer, on which further analysis was carried out. Base-line correction was carried out by subtracting the average of the last 50 channels. Decay parameters were extracted from the decay curves by the method of moments (Dyson and Isenberg, 1971). For 11 different preparations of Tb-TnC, our method yielded a mean lifetime of 1.31 ms, with a standard deviation of 0.01 ms.

**Results and Discussion**

When excited via Tyr-109, the luminescence decay curves for both Tb-TnC and Tb2-TnC are unexponential with nearly identical lifetimes of 1.31 and 1.32 ms, respectively (Fig. 2 and Table I). In Tb2-TnC, both sites III and IV are occupied by \( \text{Tb}^{3+} \); while Tb-TnC contains a mixture of species with only site III occupied, only site IV occupied, both sites occupied, and neither site occupied. Tyr-109 is located within the peptide sequence that constitutes site III (Collins et al., 1973). Our observed unexponential decay characteristics would be consistent with Tyr-109 transferring energy only to \( \text{Tb}^{3+} \) bound at site III. However, inspection of the proposed three-dimensional model of TnC (Kretsinger and Barry, 1975) reveals that the phenol moiety of Tyr-109 is close to site IV as well, such that Tyr-109 can transfer energy to \( \text{Tb}^{3+} \) bound at both sites simultaneously. If so, then the observation of unexponential decay for both Tb-TnC and Tb2-TnC suggests that the environments at the two high affinity sites of TnC are identical and give rise to identical bound \( \text{Tb}^{3+} \) lifetimes.

For Tb-Nd-TnC, the decay curve is distinctly nonexponential (Fig. 2). A method of moments analysis using two exponentials yielded lifetimes of 1.38 and 0.59 ms (Table I). The two lifetimes can be interpreted in terms of the two ions distributing themselves between sites III and IV, giving rise to the four species shown in Table II. Of the four, species (iv) is nonluminescent since \( \text{Nd}^{3+} \) is nonluminescent. The lifetime of species (i) should be similar to that of either Tb-TnC or Tb2-TnC, viz. 1.31 ms. The \( \text{Tb}^{3+} \) ions in both species (ii) and (iii) would undergo energy transfer to the \( \text{Nd}^{3+} \) ions and would, therefore, luminesce with lifetimes shorter than 1.31 ms. Furthermore, since the interionic distance in species (ii) and (iii) is identical, the extent of energy transfer and the lifetimes of the two species would also be identical. Based on this interpretation, we assign the 1.38-ms lifetime to species (i) and the 0.59-ms lifetime to species (ii) and (iii).

The amplitudes of the two decay components for Tb-Nd-TnC are nearly equal (Table I). The amplitude of a luminescence decay component is proportional to the product of the concentration of the luminescent species and the probability of that species being excited. Thus,

\[ A_1 = C_{11} P_{11} + C_{14} P_{1V} \]

and

\[ A_2 = C_{11} P_{11} + C_{14} P_{1V} \]

where \( A_1 \) and \( A_2 \) are amplitudes of the long and short lived components, respectively. \( C \) values are concentrations of \( \text{Tb}^{3+} \) bound at a given site, and \( P \) values are proportional to the probability that a bound \( \text{Tb}^{3+} \) is excited via energy transfer.
Distance between Sites in Troponin-C

Table I
Luminescence decay data and energy transfer parameters for Tb-Ln-TnC complexes

<table>
<thead>
<tr>
<th>Samples</th>
<th>Amplitude lifetime</th>
<th>Amplitude lifetime</th>
<th>Mean y squared</th>
<th>Transfer efficiency (E)</th>
<th>Critical transfer distance (R0)</th>
<th>Separation distance (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-TnC</td>
<td>1.00 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>1.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-TnC</td>
<td>1.00 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>1.58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-La-TnC</td>
<td>1.00 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>3.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-Dy-TnC</td>
<td>1.00 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>1.51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-Tb-TnC</td>
<td>0.99 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-Pr-TnC</td>
<td>0.37 ± 0.01</td>
<td>0.63 ± 0.01</td>
<td>0.52</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tb-Er-TnC</td>
<td>0.25 ± 0.01</td>
<td>0.75 ± 0.01</td>
<td>0.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-Ho-TnC</td>
<td>0.75 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>1.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-Nd-TnC</td>
<td>0.54 ± 0.01</td>
<td>0.46 ± 0.01</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-Tr-TnC</td>
<td>1.00 ± 0.01</td>
<td>0.41 ± 0.01</td>
<td>2.38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb-Nd-Tr-TnC</td>
<td>0.59 ± 0.01</td>
<td>1.39 ± 0.01</td>
<td>0.93</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Table II
Distribution of lanthanide ions between the two high affinity sites of TnC in a 1:1 mixture of Tb³⁺, Nd³⁺, and TnC

<table>
<thead>
<tr>
<th>Species</th>
<th>Site III</th>
<th>Site IV</th>
<th>Relative proportion</th>
<th>Expected relative amplitude</th>
<th>Expected luminescence lifetime</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tb³⁺</td>
<td>25</td>
<td>50</td>
<td>Same as Tb³⁺TnC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb³⁺</td>
<td>25</td>
<td>50</td>
<td>Same as Tb³⁺TnC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nd³⁺</td>
<td>25</td>
<td>50</td>
<td>Same as Tb³⁺TnC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nd³⁺</td>
<td>25</td>
<td>50</td>
<td>Same as Tb³⁺TnC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Assuming that Tb³⁺ and Nd³⁺ have the same affinity toward the two sites.

* Assuming that Tyr-109 transfers energy to Tb³⁺ bound at sites III and IV to the same extent. See text for discussion of other possibilities.

from Tyr-109; the subscripts III and IV refer to the two high affinity sites; the superscripts i, ii, and iii refer to the various species in Table II. It is known that the affinities of the two sites for Tb³⁺ are the same (Wang et al., 1981) and that Tb³⁺ and Nd³⁺ have equal affinities for the two sites. This yields C₁₁ = C₁₂ = C₂₁ = C₂₂ = C, and A₁ = A₂ = C(P₁₁ + P₁₂) regardless of the relative magnitudes of P₁₁ and P₁₂. Thus our finding here that the amplitudes of the two decay components are equal is consistent with results obtained from binding studies on the affinities of Tb³⁺ and Nd³⁺ for the high affinity sites of TnC.

Our analysis shows that our observations cannot resolve the question of whether Tyr-109 transfers energy only to Tb³⁺ bound at site III, or at site IV, or both. However, the assignment of the lifetimes is independent of this question. Thus, taking the lifetime of Tb-TnC (1.31 ms) as τ₁ and the short lived lifetime of Tb-Nd-TnC (0.59 ms) as τ₂, an energy transfer efficiency of 55.2% was obtained (Equation 1). Taking 0.93 ms as the critical transfer distance R₀ for the Tb³⁺-Nd³⁺ couple (Horrocks et al., 1980), we obtained 0.90 nm as the distance r between the two high affinity sites of TnC.

E and r for energy transfer from Tb³⁺ to other lanthanide ion acceptors were obtained in a similar manner and are presented in Table I. Note that for certain acceptors such as La³⁺ and Dy³⁺, R₀ is so small that no transfer takes place; the decays of these Tb-Ln-TnC complexes are essentially identical with that of Tb-TnC. For weak acceptors such as Pr³⁺ and Eu³⁺, a small amount of energy transfer takes place, causing a small deviation from single exponential decay. The analyses of these decay curves are relatively difficult and subject to a certain amount of error because τ₁, is not very different from τ₂. The magnitude of this error in the lifetime determinations can be appreciated by noting that the lifetimes of the long lived component for Tb-Pr-TnC and Tb-Er-TnC deviate from 1.31 ms by ~0.3 ms, representing an error of ~20%. For this reason, we do not consider distance measurements using these samples to be very reliable. Nevertheless, using 1.31 ms as τ₁ and the shorter lifetimes as τ₂, the values of r obtained for these samples are in reasonable agreement with each other and with that obtained for Tb-Ho-TnC and Tb-Nd-TnC. We consider r obtained from these latter two samples to be the most reliable because there are no problems with the analysis since the longer and shorter lifetimes differ from each other significantly. Consequently, we take the distance to be the average of these two determinations, viz. 0.92 nm, with an estimated uncertainty of 0.02 nm.

Entirely similar results were obtained when the tryptic fragment TnC, which contains only sites III and IV, was used instead of intact TnC (Table I), supporting the notion that this fragment largely retains the native conformation (Leavis et al., 1978).

It should be noted that attempts at quantifying the energy transfer efficiencies by steady state measurements were unsuccessful for the following reason. The luminescence intensity of Tb-TnC was depressed upon the addition of any lanthanide ion, including La³⁺, which is known to be incapable of accepting energy from Tb³⁺ owing to poor spectral overlap. Most likely, the presence of a second Ln³⁺ induces a slight structural change that alters the energy transfer efficiency between Tyr-109 and the bound Tb³⁺. Our lifetime measure-
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ments, therefore, represent the only viable method for studying intermetal energy transfer in TnC.

The distance obtained here (0.92 nm) is in excellent agreement with the distance between the two calcium-binding sites in parvalbumin obtained by energy transfer measurements (0.94 nm) (Horrocks et al., 1980; Rhee et al., 1981). It is, however, somewhat shorter than the same distance in parvalbumin obtained from the x-ray crystallographic structure (1.18 nm) (Kretsinger and Nockholds, 1973). This discrepancy may be due to a systematic error in the determinations of $R_0$ (Rhee et al., 1981) or to the aforementioned structural alteration. Based on sequence homology and symmetry arguments, Kret- singer and Barry (1975) had proposed a model of TnC that is composed of two parvalbumin-like halves. In view of the identification of sites I and II as the low affinity sites and sites III and IV as the high affinity sites, pairs of the same affinity would be in the same half of the TnC molecule. In the above model, the distance between sites III and IV is 1.12 nm, whereas the distance between III and I, for instance, is 3.0 nm. The distance obtained in this work is much closer to the former value. Thus, our result is consistent with the Kretsin- ger and Barry model and with the localization of the two high affinity sites within one of the two parvalbumin-like domains.

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