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Fluorescence Energy Transfer Studies of Skeletal Troponin C Proximity between Methionine-25 and Cysteine-98†

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ABSTRACT: The distance between two specific residues in skeletal troponin C was measured by the method of fluorescence energy transfer. Dansylaziridine attached to Met-25 was used as the energy donor and 5-(iodoacetamido)eosin attached to Cys-98 as the acceptor. The transfer efficiency was determined from quenching of donor intensity. An efficiency of 66% was obtained for Ca^{2+}-free troponin C and 81% for the fully saturated Ca^{2+} complex. From these results and depolarization data obtained from donor- and acceptor-labeled proteins, a range of the donor–acceptor distance (R) was calculated by the dynamic averaging method [Dale, R. E., Eisinger, J., & Blumberg, W. E. (1979) *Biophys. J. 26*, 161]. The ranges were 29–51, 26–51, and 26–51 Å for Ca^{2+}-free troponin C, the half-saturated Ca^{2+} complex, and fully saturated Ca^{2+} complex, respectively. These results suggest that Ca^{2+} binding may induce a very small reduction in the minimum donor–acceptor distance but does not produce any global deformation of troponin C. Qualitatively similar conclusions were obtained with 5-(iodoacetamido)fluorescein attached to Cys-98 as the acceptor. A rotational correlation time of 6.3 ns was determined by the nanosecond method for Ca^{2+}-free troponin C which was labeled with *N*-(iodoacetyl)-*N'*-(1-sulfo-5-naphthyl)ethylenediamine at Cys-98. While a reduction of about 1 ns in the correlation time was observed for the Ca^{2+} complexes, the change was too small to warrant any conclusion of a change in the overall shape of the protein induced by Ca^{2+} binding. The relatively large minimum distance between donor and acceptor sites suggests that structural perturbations initiated by Ca^{2+} binding to the high-affinity sites would be transmitted over a considerable distance to the attached donor probe.

While the molecular mechanism by which Ca^{2+} binding to troponin modulates actomyosin ATPase has not been fully elucidated, it is generally accepted that the binding triggers a series of reversible molecular changes which are propagated across the thin filament structure. The transfer of this structural information to specific sites on the actin filament results in the activation of the actin–myosin interaction. Since the stoichiometry of troponin:tropomyosin:actin is 1:1:7, the transfer of structural information which is induced by Ca^{2+} binding must occur over a large distance. Our present understanding of the transfer mechanism is poor because of the lack of detailed structural information.

Of the three subunits of troponin, TNC[1] is the Ca^{2+} receptor. The other two subunits, troponin T and troponin I, provide a link to tropomyosin and actin, and together they are thought to exert the inhibitory effect via tropomyosin on the actin–myosin interaction. The four Ca^{2+} binding sites of TNC can be divided into two classes according to their affinity for Ca^{2+}. The two low-affinity sites (sites 1 and 2) are Ca^{2+} specific, and the two high-affinity sites (sites 3 and 4) also bind Mg^{2+} competitively. The affinity of the two classes of sites for Ca^{2+} differs by about 2 orders of magnitude, with the affinity of the Ca^{2+} specific sites being about 3×10^5 M^{-1} (Potter & Gergely, 1975).

The three-dimensional structure of rabbit skeletal TNC has not yet been determined, although its amino acid sequence has been known for some time (Collins et al., 1977). By analogy with the primary structure of a parvalbumin, four homologous regions have been postulated as the Ca^{2+} binding sites of TNC (Kretsinger & Barry, 1975). Subsequent studies including those with cyanogen bromide and proteolytic fragments (Potter et al., 1976; Leavis et al., 1978) have established that the two regions corresponding to the Ca^{2+}-specific sites are located in the N-terminal half of the molecule and the other two regions corresponding to the Ca^{2+}–Mg^{2+} high-affinity sites are at the C-terminal half. Many spectroscopic studies have shown that Ca^{2+} binding to the high-affinity Ca^{2+}–Mg^{2+} sites produces large conformational changes in the C-terminal half of the molecule, as reflected by an increase in tyrosyl fluorescence (van Eerd & Kawasaki, 1972) and α helicity (Kawasaki & van Eerd, 1972), spectral shifts in the UV absorption curve (Head & Perry, 1974), and an enhancement of the fluorescence of extrinsic probes attached to Cys-98 (Potter et al.,

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[1] Abbreviations: TNC, Ca^{2+} binding subunit of troponin; TNT, tropomyosin binding subunit of troponin; TNI, ATPase inhibitory subunit of troponin; DNZ, dansylaziridine; IAE, 5-(iodoacetamido)eosin; IAF, 5-(iodoacetamido)fluorescein; IAEDANS, *N*-(iodoacetyl)-*N'*-(1-sulfo-5-naphthyl)ethylenediamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TNC-DNZ, TNC labeled with DNZ; TNC-IAE, TNC labeled with IAE; TNC-IAF, TNC labeled with IAF; TNC-DNZ-IAE, TNC doubly labeled with DNZ and IAE; TNC-DNZ-IAF, TNC doubly labeled with DNZ and IAF; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol.

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1976; Cheung, 1977; Cheung & Garland, 1978). Structural changes that are induced by Ca^{2+} binding to the low-affinity sites have also been reported by proton NMR (Levine et al., 1977; Seamon et al., 1977) and extrinsic fluorescence (Potter et al., 1976; Johnson et al., 1978; Cheung & Garland, 1978). Of particular interest among the several fluorescent probes that have been used to study TNC is dansylaziridine, which was attached to Met-25 under saturating Ca^{2+} conditions (Johnson et al., 1978). This probe when attached to TNC responds to Ca^{2+} binding to the low-affinity sites with large spectral changes that are in the opposite direction of the small changes induced by Ca^{2+} binding to the high-affinity sites.

In the present study, we estimated the intramolecular distance of TNC between Met-25 and Cys-98 by resonance energy transfer with dansylaziridine attached to the methionine as the energy donor and 5-(iodoacetamido)eosin or 5-(iodoacetamido)fluorescein attached to the other residue as the acceptor. Because of the usual ambiguity of the orientation factor between donor and acceptor dipoles, we carried out fluorescence depolarization measurements on individual donor- and acceptor-labeled proteins and applied the approach of Dale & Eisinger (1974) to determine a range of the donor–acceptor distance. The range was considerably narrowed on the basis of measured rotational correlation times and other structural information. This measured intramolecular distance provides a basis for proximity mapping of TNC and troponin reconstituted from labeled subunits.

Materials and Methods

Reagents. The fluorescent probe IAEDANS was obtained from Aldrich Chemical Co. and IAE and IAF were from Molecular Probes, Inc. Ultrapure enzyme grade urea was used for protein isolation. A standard solution of Ca^{2+} (0.10 M) was purchased from Orion, Inc. All other chemicals were of reagent grade.

Protein Preparations. Troponin was prepared from rabbit skeletal muscle by a slight modification of the Ebashi method (Ebashi et al., 1971). Troponin C was isolated from the troponin by DEAE-cellulose chromatography in the presence of 6 M urea (Perry & Cole, 1974). The isolated protein was dialyzed against 0.1 M KCl, 0.2 mM DTT, 2 mM EGTA, and 20 mM Tris at pH 7.5. The purified protein was judged to be homogeneous by the criterion of sodium dodecyl sulfate–polyacrylamide gel electrophoresis. TNC concentration was determined by UV absorbance by using the extinction coefficient $E^{1\%}_{277} = 2.3$ (Murray & Kay, 1972) and a molecular weight of 18 000. Sulfhydryl content was determined spectrophotometrically by using DTNB (Ellman, 1959).

Prior to labeling, TNC (ca. 100 μM) was first dialyzed against the EGTA buffer which contained no DTT. Labeling with the various fluorescent probes was carried out in the dark and at room temperature for 20–24 h by using a 1.2–1.4-fold molar excess of the probes immediately after dialysis. The reactions with the three probes IAEDANS, IAE, and IAF were effected in the absence of Ca^{2+}. DNZ and IAF were first dissolved in alcohol prior to addition to TNC. The volume of the organic solutions (5–10 mM) was kept below 1–2% of the total volume. A fresh solution of DNZ (10 mM) in alcohol was used to label TNC under saturating Ca^{2+} conditions (2 mM EGTA plus 2.5 mM Ca^{2+}) as previously described (Johnson et al., 1978). Prior saturation of the Ca^{2+} binding sites was necessary in order to direct DNZ to Met-25 with high degree of labeling. Modification of Cys-98 with IAE was carried out in the presence of 2 mM EGTA essentially as described by Cherry (1978). A similar procedure was used to label TNC with IAF. Unreacted probes were removed by exhaustive dialysis against 0.1 M KCl, 2 mM EGTA, and 20 mM Tris at pH 7.5. When both DNZ and IAE (or IAF) were introduced into the same TNC molecule, the protein was first labeled with DNZ, followed by exhaustive dialysis to remove bound Ca^{2+} in the presence of DTT. The DNZ-labeled protein was then further dialyzed against the EGTA buffer but without DTT before modification with a second probe.

Incorporation of the probes into TNC was determined spectrophotometrically by using the following extinction coefficients: IAEDANS, $\epsilon = 6.0 \times 10^3$ M^{-1} cm^{-1} at 337 nm (Hudson & Weber, 1973); IAE, $\epsilon = 8.3 \times 10^4$ M^{-1} cm^{-1} at 528 nm (Cherry, 1978); IAF, $\epsilon = 7.7 \times 10^4$ M^{-1} cm^{-1} at 496 nm (Takashi, 1979); DNZ, $\epsilon = 3.98 \times 10^3$ M^{-1} cm^{-1} at 350 nm (Johnson et al., 1978). Whenever necessary, the absorbance of TNC at 277 nm was corrected for probe absorption. The correction terms were estimated from the absorption spectra of free probes. These values were $0.17A_{peak}$ for IAEDANS, $0.22A_{peak}$ for IAE, and $0.24A_{peak}$ for IAF, and $0.463A_{peak}$ for DNZ, where A_{peak} is the peak absorbance of the attached probe in a given sample. A total of six TNC conjugates were prepared for the present study: TNC-DNZ, TNC-AEDANS, TNC-IAE, TNC-IAF, TNC-DNZ-IAE, and TNC-DNZ-IAF. In the two doubly labeled proteins DNZ was the energy donor, and the other probe was the energy acceptor.

Tryptic digestion of TNC labeled with IAE was carried out as described by Leavis et al. (1978). The resulting fragments were separated on 8% polyacrylamide containing 5 M urea and 0.1 mM Ca^{2+} and extracted from the gels as previously described (Leavis et al., 1978).

Free Ca^{2+} concentration was calculated from the amount of Ca^{2+} added by using the computer program of Perrin & Sayce (1967) with appropriate binding constants for Ca^{2+} and H^+ to EGTA (Johnson et al., 1978). Since a relatively high EGTA level (2 mM) was used to adequately control the level of free Ca^{2+}, it was necessary to restore the pH to 7.5 with small volumes (microliters) of KOH after Ca^{2+} was added to the TNC solutions.

Spectroscopic Measurements. Steady-state fluorescence measurements were made in a Perkin-Elmer 650-40 ratio fluorescence spectrometer thermostated at 20.0 ± 0.1 °C. An initial volume of 1.0 mL was used in most measurements. The quantum yield of DNZ attached to TNC was determined by using the comparative method (Parker & Reese, 1960) with quinine sulfate in 0.1 N H_2SO_4 as the standard. A value of 0.70 was used as the quantum yield of the standard at 25 °C (Scott et al., 1970). The response of the instrument detector system was found to be reasonably flat in the region 350–620 nm, making it unnecessary to use corrected emission spectra for quantum yield comparison. Areas of emission spectra were determined with a planimeter. The apparent quantum yield was corrected for the effect of depolarization due to Brownian rotational motion by using the limiting anisotropy of the attached probe (Shinitzky, 1972). Steady-state polarization was determined in the Perkin-Elmer instrument with a Polaroid HNP'B polarizer assembly. Standard corrections were made for instrumental depolarization as previously described (Cheung et al., 1971).

A photon-counting pulse fluorometer (Harvey & Cheung, 1977) was used to determine the lifetime of IAEDANS attached to TNC. Samples were excited with vertically polarized light (337 nm), and the emission was isolated at a right angle with a polarizer oriented at the magic angle of 54° with respect to the vertical direction and Corning filters 3-144 and 3-72 in tandem. A nonlinear least-squares search was used to

deconvolute the decay data (Garland et al., 1980). The response function was obtained with 2,5-diphenyloxazole (PPO) in ethanol as a standard substance (Wahl et al., 1974). Rotational correlation times of the TNC-IAEDANS conjugate were determined by analyzing the decay function $F_{\parallel}(t)$, which is given by

$$F_{\parallel}(t) = [\sum_i A_i \exp(-t/\tau_i)][1 + 2\sum_j r_{ij} \exp(-t/\phi_{ij})] \quad (1)$$

In this equation, τ_i is the fluorescence lifetimes of the fluorophore, and ϕ_{ij} is the rotational correlational times of the protein–fluorophore conjugate. Since τ_{ij}'s were independently determined in a separate experiment (see above), ϕ_{ij} could be extracted from the observed $F_{\parallel}(t)$ from eq 1 (Rigler & Ehrenberg, 1976).

Two different methods were used to determine the limiting anisotropy of TNC-DNZ and TNC-IAE. The anisotropy decay method was used to obtain r_0 for TNC-DNZ, as previously described (Liu et al., 1981). For this purpose, samples were excited at 337 nm, and the emission was isolated with Corning filters 3-144 and 3-72. The steady-state method was used to determine r_0 for TNC-IAE by using sucrose to increase solvent viscosity.

Determination of Donor–Acceptor Distance. Transfer of resonance energy from donor to acceptor was determined from the quenching of steady-state donor intensity. Acceptor sensitization was also measured but only to confirm resonance energy transfer as the predominant donor quenching mechanism. For each set of experiments, three labeled proteins were required: donor-labeled TNC (TNC-DNZ), acceptor-labeled TNC (TNC-IAE or TNC-IAF), and donor–acceptor-labeled TNC (TNC-DNZ-IAE or TNC-DNZ-IAF). Because of nonstoichiometric labeling by both donor and acceptor, the transfer efficiency E was calculated from the following relationship:

$$E = 1 - \frac{F_{da} - F_d(1 - f_a)f_d}{F_d} \quad (2)$$

F_d is the emission intensity of the donor in the singly labeled protein (TNC-DNZ), F_{da} is the donor intensity in the doubly labeled protein (TNC-DNZ-IAE or TNC-DNZ-IAF), and f_d and f_a are the fractional occupancy of the donor and acceptor sites in the doubly labeled proteins, respectively. Both F_d and F_{da} were measured with excitation at 340 nm (5-nm spectral bandwidth) and emission at 2-nm intervals from 430 to 470 nm (10-nm spectral bandwidth). The average of the results obtained at the several emission wavelengths was used to calculate E.

The Förster critical transfer distance was calculated from

$$R_0^6 = (8.79 \times 10^{-5}) n^{-4} Q_d \kappa^2 J \ (\text{Å}^6) \quad (3)$$

where n is the refractive index and was taken as 1.40, Q_d is the donor quantum yield, κ^2 is the orientation factor, and J is the spectral overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor. J was approximated by the summation

$$J = \frac{\sum F_d(\lambda)\epsilon_a(\lambda)\lambda^4\Delta\lambda}{\sum F_d(\lambda)\Delta\lambda} \quad (4)$$

$F_d(\lambda)$ is the fluorescence spectrum of the donor in TNC-DNZ expressed in an arbitrary unit, and $\epsilon_a(\lambda)$ is the extinction coefficient of the acceptor in TNC-acceptor expressed in M^{-1} cm^{-1}. The summation was taken over 2-nm intervals. The distance R between the donor and acceptor in the doubly labeled protein was calculated from

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (5)$$

The orientation factor κ^2 may assume values from 0 to 4.0. If both donor and acceptor dipoles are isotropic and have motional freedom that is fast compared to the transfer time (dynamic averaging), $\kappa^2 = {}^2/_3$. Since this factor is difficult to determine with certainty, several methods have been proposed for obtaining an estimate. If the isotropic condition is not met but the dynamic averaging condition is satisfied, upper and lower bounds for κ^2, and hence for R, may be obtained from experimentally determined donor and acceptor depolarizations (Dale et al., 1979). This method was followed in the present work in order to bracket the donor–acceptor distance.

Results and Discussion

Labeling of TNC. Under optimal conditions the three probes IAEDANS, IAE, and IAF were incorporated into TNC with the following degrees of labeling: IAEDANS, 0.85–0.95; IAE, 0.60–0.99; IAF, 0.70–0.86. The extent of incorporation of IAE and IAF was independent of prior labeling of the protein with DNZ. The degree of labeling was low with old TNC solutions which had not been properly dialyzed against DTT as prescribed under Materials and Methods. When these conjugates with low degrees of labeling were dialyzed against DTT and subsequently relabeled, a high degree of labeling was always obtained. A typical TNC-IAEDANS preparation contained 0.86 mol of label per mol of protein. Sulfhydryl titration of this sample with DTNB indicated the presence of only 0.1 free sulfhydryl per TNC. Since the single sulfhydryl of TNC is most reactive with DTNB (Potter et al., 1976), these results suggest that the loss of titratable SH in TNC-IAEDANS was likely due to its modification by the fluorescent probe. Similar SH titration results were also obtained with TNC-IAE and TNC-IAF. Taken together, the results show that Cys-98 was selectively labeled by all three reagents in the absence of Ca^{2+}. When TNC-IAE was tryptic digested, two fragments were separated from acrylamide gel electrophoresis corresponding to the TR1 and TR2 fragments (Leavis et al., 1978). Analysis of the fragments extracted from the gels showed a typical and intense eosin emission spectrum in the TR2 fraction. This is compatible with labeling of Cys-98 since TR2 contains this residue.

Although DNZ is known to react with sulfhydryls (Scouten et al., 1974), it was shown to react with the Ca^{2+} complex of TNC predominantly at Met-25 rather than Cys-98 (Johnson et al., 1978). When the degree of labeling was kept low, the label was exclusively localized on the tryptic T3 fragment (residues 21–37) as demonstrated by partial peptide maps. The optimal quantum yield change induced by Ca^{2+} arose from the label incorporated into T3, and the label attached to other sites apparently did not respond with spectral changes to Ca^{2+} binding to the Ca^{2+}-specific sites. In the present work, DNZ was incorporated into TNC in the presence of saturating Ca^{2+} to the extent of 0.40–0.97 mol/mol of protein. Emission measurements showed that the attached DNZ responded to addition of Ca^{2+} or Mg^{2+} in a characteristic way as was previously reported (Johnson et al., 1978). The probe quantum yield decreased by about 10% together with a red shift of the emission maximum from 530 to 535 nm upon addition of a large excess of Mg^{2+} or less than 2 mol of Ca^{2+}. Under saturating Ca^{2+} conditions, the quantum yield was enhanced by a factor of 1.9–2.2 with a concomitant blue shift of about 12 nm from 530 to 518 nm. The observed spectral changes were characteristic of the optimal changes due to the probe

Table I: Fluorescence Parameters of TNC Labeled with IAEDANS at Cys-98[a]

cation/TNC				lifetime			correlation time	
Mg^{2+}	Ca^{2+}	λ_{em} (nm)	rel Q	τ_1 (ns)	τ_2 (ns)	χ_R^2	ϕ (ns)	χ_R^2
0	0	485	1.00	16.60 ± 0.58	8.14 ± 0.83	1.8	6.3	2.3
0	2	480	1.29	15.21 ± 0.10		2.0	5.1	2.0
0	>4	480	1.30	15.22 ± 0.13		1.9	5.3	1.9
>2	0	480	1.29					

[a] Protein concentration was 20 μM. For the sample fully saturated with Ca^{2+} (>4 Ca^{2+}), EGTA was 2.0 mM and Ca^{2+} was 2.5 mM. When Mg^{2+} was present, Mg^{2+}/TNC was ca. 1000. Excitation wavelength was 340 nm for steady-state spectral measurements. Relative quantum yield (rel Q) was taken as 1.0 for the cation-free sample. The fractional amplitudes associated with the two lifetimes τ_1 and τ_2 are 0.62 and 0.38, respectively, when cation was absent. Only one lifetime was observed when cation was present. The estimated error in the rotational correlation time ϕ is about 0.5 ns.

incorporated into T3 (Johnson et al., 1978). If a significant amount of DNZ was attached to another site, the change in quantum yield induced by Ca^{2+} would be less than optimal. These results suggest that in the experiments reported here the probe was attached predominantly to a single site. That the single sulfhydryl was not significantly labeled by DNZ in the presence of Ca^{2+} was confirmed by the finding that TNC-DNZ was essentially fully reactive with DTNB as was unmodified TNC when Ca^{2+} was excluded in the assay medium.

As already indicated, IAE or IAF was incorporated into TNC equally well regardless of whether the protein had been modified by DNZ. In these doubly labeled proteins (TNC-DNZ-IAE or TNC-DNZ-IAF), the sulfhydryl content was reduced to about 0.1 or less per TNC, showing that the eosin or fluorescein moiety was located at Cys-98.

TNC-IAEDANS Conjugates. The fluorescence emission properties of TNC labeled with IAEDANS are summarized in Table I. There was a 30% enhancement of the probe quantum yield upon addition of either Mg^{2+} or Ca^{2+}. This increase was accompanied by a 5-nm blue shift of the emission peak. The spectral changes elicited by Ca^{2+} addition were the same whether 2 mol or larger excess of Ca^{2+} were added per mol of protein. The Ca^{2+}-free conjugate showed two lifetimes, 8 and 6 ns. Addition of either Mg^{2+} or Ca^{2+} eliminated the short component, and the long component was identical for both the half-saturated and fully saturated Ca^{2+} complexes. It is evident that IAEDANS attached to Cys-98 is not sensitive to Ca^{2+} binding to the low-affinity, Ca^{2+}-specific sites. To ascertain the validity of the two-component emission, we analyzed the decay data by two different procedures, both based on a nonlinear least-squares search. The first was the traditional method where lamp profiles were obtained with a scattering agent, and these lamp pulses were used for data deconvolution. The other was the procedure indicated under Materials and Methods in which the lamp pulses were calculated from a standard (PPO) whose single lifetime was independently known. Since both methods yielded the same results with acceptable fitting statistics, we conclude that the two-component characteristic was an inherent property of the system. The origin of this multiple emission is not clear. While it might be tempting to speculate the presence of two conformations of TNC, any such speculation cannot be justified until additional data are available.

We derived from the observed decay of the polarized emission $F_\parallel(t)$ a single rotational correlation time of about 6 ns for the apoprotein. This value is in good agreement with the 7.3 ns previously reported (Johnson et al., 1978) with the anisotropy decay procedure and with DNZ attached to Met-25. A small decrease of about 1 ns was observed for the fully saturated Ca^{2+} complex. This small difference cannot be regarded as significant since the estimated error in the

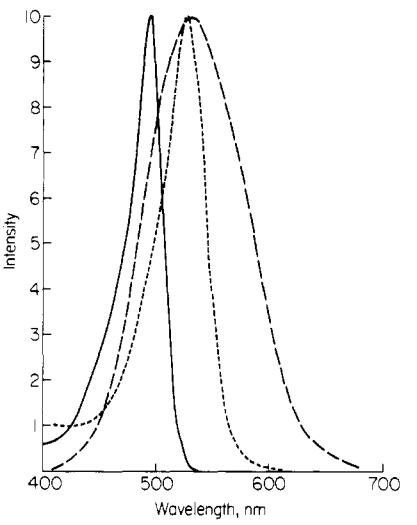

FIGURE 1: Spectral overlap of donor emission with acceptor absorption. Emission spectrum: DNZ attached to TNC (···). Absorption spectra: IAF attached to TNC (—); IAE attached to TNC (---). These spectra were normalized to the peak intensity.

Table II: Fluorescence Parameters of TNC Labeled with IAE at Cys-98[a]

Mg^{2+}/TNC	Ca^{2+}/TNC	λ_{em} (nm)	rel Q
0	0	556	1.00
0	2	558	1.20
0	>4	560	1.61
>2	0	558	1.07

[a] Protein concentration was 10 μM, excitation wavelength was 520 nm, and relative cation concentrations were similar to those listed for Table I.

measured correlation time was about 0.5 ns. An equivalent rigid rotor with a 20% hydration is expected to have a rotational correlation time of about 5 ns at 20 °C and a diameter of 36 Å. Our results suggest that TNC is relatively symmetric and that Ca^{2+} binding does not induce any significant distortion of the overall molecular symmetry. The procedure which we used to obtain the rotational correlation time differs from the usual method used in many reported studies in which the correlation time is derived from anisotropy decay plots. The advantage of the present method is that multiple lifetimes are explicitly taken into account in the analysis of $F_\parallel(t)$ (Rigler & Ehrenberg, 1976). In the present case, known values of τ_i were used in fitting the observed $F_\parallel(t)$ to eq 1. The single-fitted values of ϕ are statistically acceptable as judged by their χ_R^2 values.

TNC-IAE Conjugate. TNC-IAE emitted maximally at 556 nm upon excitation with either 340- or 520-nm radiation (Figure 1). This probe was found to be sensitive to both Mg^{2+}

Table III: Fluorescence Energy Transfer Parameters in Troponin C[a]

conditions and parameter	sample					
	TNC-DNZ			TNC-DNZ-IAE		
Ca^{2+}/TNC	0	2	4	0	2	4
E				0.66	0.67	0.81
Q				0.144	0.130	0.300
$J \times 10^{-15}$ (M^{-1} cm^{-1} nm^4)				2.98	2.98	3.10
limiting anisotropy, r_0						
donor	0.156	0.197	0.200	0.156	0.197	0.200
acceptor				0.324	0.336	0.344
fundamental anisotropy, r_f						
donor	0.247[b]	0.247	0.247	0.247	0.247	0.247
acceptor				0.400[c]	0.400	0.400
depolarization factors						
$\langle d_D^X \rangle$	0.795	0.893	0.900	0.795	0.893	0.900
$\langle d_A^X \rangle$				0.900	0.917	0.927
cone half-angle (θ) (deg)	31.2	21.8	21.8	21.8	19.3	17.2
orientation factors						
κ^2(min)				0.102	0.063	0.058
κ^2(max)				3.2	3.5	3.6
Förster critical distance (Å)						
$R_0(^2/_3)$				43.8	43.6	49.3
R_0(min)				31.9	29.4	33.0
R_0(max)				57.0	57.4	65.4
donor–acceptor distance (Å)						
$R(^2/_3)$				39.0	38.6	38.8
R(min)				28.5	26.0	25.8
R(max)				50.7	50.9	51.3

[a] Donor, dansylaziridine (DNZ) at Met-25; acceptor, (iodoacetamido)eosin (IAE) at Cys-98. [b] Chen (1967). [c] Cherry & Schneider (1976).

binding and Ca^{2+} binding to the protein (Table II). A 20% increase in quantum yield was observed upon addition of slightly less than 2-fold excess of Ca^{2+}. A small, but reproducible, red shift of the emission peak was also observed. Addition of Ca^{2+} to beyond 4-fold excess resulted in a further increase to a final enhancement of about 60% and a final red shift of about 4 nm. The spectral shift induced by 2 mol of Ca^{2+} was similar to that induced by Mg^{2+}, although the quantum yield change induced by Mg^{2+} was smaller. If the initial quantum yield increase resulted from Ca^{2+} binding to the Mg^{2+}–Ca^{2+} high-affinity sites, then the additional increase to 60% must reflect binding of Ca^{2+} to the low-affinity, Ca^{2+} specific sites. These results are in contrast with those obtained from TNC-IAEDANS, which did not show Ca^{2+} sensitivity beyond 2 mol of Ca^{2+} per mol of protein.

Energy-Transfer Studies of TNC-DNZ-IAE. The absorption spectra of TNC labeled with IAE and IAF (TNC-IAE and TNC-IAF) and fluorescence emission spectrum of the protein modified by DNZ (TNC-DNZ) are shown in Figure 1. These curves illustrate the spectral overlap of the DNZ emission with the absorption of the other two probes and show that IAE and IAF should be good acceptors of excitation energy from DNZ. The quenching of the donor emission resulting from introduction of an eosin acceptor into the same TNC molecule is depicted in Figure 2. When the spectrum of the doubly labeled protein is compared with the spectra of the singly labeled proteins, it is clear that upon excitation at 340 nm, the doubly labeled protein emits in two regions of the spectrum, one corresponding to the emission of DNZ and the other to that of IAE. From the individual emission curves of TNC-DNZ and TNC-IAE, it can be seen that the acceptor IAE has little or negligible emission below about 470 nm where the donor DNZ still emits strongly. This is the basis for measuring donor quenching in the doubly labeled protein at wavelengths below 470 nm. This choice of wavelengths minimizes complications arising from acceptor fluorescence. In Figure 2 the concentrations of both donor and acceptor in the individual singly labeled proteins were normalized to those of the donor and acceptor in the doubly labeled protein. The difference in intensity between the spectra of TNC-DNZ and TNC-DNZ-IAE in the region below 525 nm represents quenching of donor emission due to the presence of an acceptor in the same TNC molecule. This decrease occurred concomitantly with an enhancement of the acceptor emission as reflected by an increase in intensity in the 560-nm region relative to TNC-DNZ and TNC-IAE. A control sample of donor-labeled TNC was prepared in which Cys-98 was modified by *N*-ethylmaleimide. Comparison of the emission spectrum of the control sample with that of TNC-DNZ revealed no significant difference (±1%) in the quantum yield between the two samples. Thus, we are confident that modification of Cys-98 did not affect the quantum yield of the attached DNZ. The observed quenching of donor emission depicted in Figure 2 must, therefore, result predominantly from Förster energy transfer.

The transfer efficiency from DNZ to IAE was evaluated from preparations of TNC with different degrees of labeling. The various spectroscopic parameters that were required to calculate the donor–acceptor distance are listed in Table III, along with the distances based on different values of the orientation factor κ^2. Also listed in Table III are the corresponding parameters determined in the presence of Ca^{2+}. As already indicated we are reasonably confident that the acceptor IAE was located at Cys-98. The site of attachment of DNZ requires additional comments. In preliminary experiments on samples with labeling stoichiometry ranging from 0.40 to 0.98, essentially identical spectral changes in response to Ca^{2+} addition were observed. The variation in quantum yield enhancement was 10% or less. The variation of the measured transfer efficiency in doubly labeled TNC-DNZ-IAF (see following section) was less than 5% from two samples with donor stiochiometry 0.40 and 0.81. These results provide some confidence that the donor labeled a major site at both low and high degree of labeling and that the measured transfer efficiency is a good representation of the transfer between two specific sites.

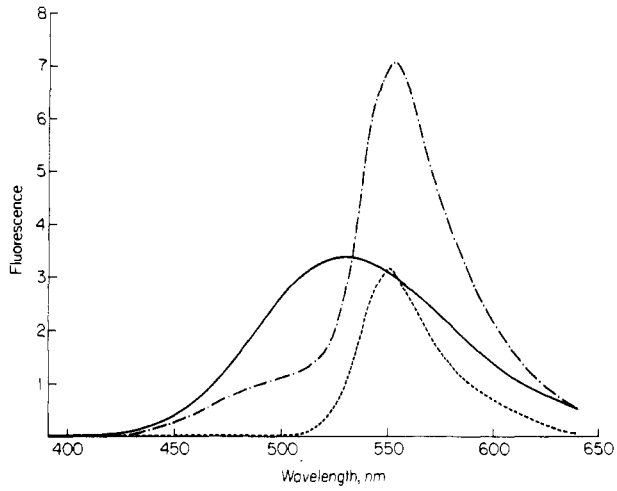

FIGURE 2: Fluorescence spectra of singly and doubly labeled TNC. Donor labeled TNC, TNC-DNZ (—); donor- and acceptor-labeled TNC, TNC-DNZ-IAE (-·-); acceptor-labeled TNC, TNC-IAE (···). TNC concentration of TNC-DNZ and TNC-DNZ-IAE was 8.5 μM.

We first consider the distance parameters based on isotropic and random orientation of donor and acceptor dipoles ($\kappa^2 = {}^2/_3$). The transfer efficiency between DNZ and IAE in TNC was 66%, and it increased to 81% when all four sites were saturated with Ca^{2+}. In spite of this increase, $R({}^2/_3)$ derived from $\kappa^2 = {}^2/_3$ remained unchanged. This is due to the fact that the donor quantum yield was approximately 2-fold enhanced in the presence of Ca^{2+}, which resulted in a 10% increase in $R_0({}^2/_3)$. These results show that an observed increase in the transfer efficiency does not necessarily imply a decrease in the donor–acceptor distance. If the use of $\kappa^2 = {}^2/_3$ were justified, then the present results would indicate that Ca^{2+} did not induce any significant global change in the donor–acceptor separation.

Earlier NMR studies (Seamon et al., 1977; Strong et al., 1977) suggested that sulfhydryl probes attached to Cys-98 of TNC had considerable motional freedom. This is corroborated in the present study since the limiting anisotropy (r_0) of IAE attached to TNC was smaller than the fundamental anisotropy (r_f) of the free probe (Table III). The attached DNZ was also considerably depolarized. From the relationship $r_0/r_f = \cos^2\theta(1 + \cos\theta)^2/4$ (Kawato et al., 1977), the half-angle (θ) of a cone within which the probe rotated was readily calculated. It is evident from the values of θ listed in Table III that the donor was considerably less restricted in the apoprotein than in the Ca^{2+} complexes. Ca^{2+} binding reduced this angular range from 31° to 22°. The acceptor had a smaller half-angle than the donor. It is noted that θ for the acceptor was also reduced by Ca^{2+} binding. These results suggest that it may be inappropriate to use a single given value of κ^2 for the donor–acceptor pair in both the apoprotein and its Ca^{2+} complexes. This conclusion is supported by the observations that Ca^{2+} binding to TNC is sensed by DNZ (Johnson et al., 1978; present results) and IAE (present work, Table II) attached to their respective sites. This sensitivity indicates that the microenvironment of a given probe is different in the apoprotein than in the Ca^{2+} complexes. To further consider the appropriate values of κ^2, we assume that (1) both donor and acceptor have restricted orientational freedom such that their dipoles do not sample all orientations with respect to the protein and (2) their segmental motion is rapid compared with donor lifetime. The method of dynamic averaging of Dale & Eisinger (1974) is applicable to such a system and can be used to determine lower and upper bounds for κ^2 if polarization data of the attached donor and acceptor are available. From the experimentally determined depolarization factors, the lower and upper bounds for κ^2 were determined. These parameters yielded the minimum and maximum values of R. For Ca^{2+}-free TNC, the range of R is 29–51 Å. This range could be reduced if data on transfer depolarization were available. Inspection of the contour plots given by Dale et al. (1979) revealed that transfer depolarization data would not increase κ^2(min) to any significant extent but would reduce the upper bound for κ^2 to yield a narrower range in R. In the absence of transfer depolarization data, the range of R given in Table III represents the best estimate derivable from the dynamic averaging method. The estimated lower limit of the range, R(min), is likely to be accurate, while the indicated upper bound R(max) may be an overestimate. Thus, we consider the minimum separation between the donor and acceptor sites to be 29 Å in Ca^{2+}-free TNC.

From the contour plots of Dale et al. (1979), we also determined that the error in R resulting from the use of $\kappa^2 = {}^2/_3$ [$R({}^2/_3)$ in Table III] instead of the indicated κ^2(min) and κ^2(max) is greater than 20%, suggesting that the appropriate distance is likely to be different from 39 Å. This consideration does not alter the estimated range of R (29–51 Å) but places the actual value to be closer to either extreme value than the midpoint of the range [which is very close to $R({}^2/_3)$]. Since TNC appears to be reasonably symmetric, its longest dimension may not be too much larger than the diameter of a spherical equivalent (36 Å). Thus, the upper limit of 51 Å may also be ruled out, leaving an estimate of R near the lower limit (29 Å). This estimate also has support from the predicted structure of TNC in which the Ca^{2+} at site 1 is separated from that at site 2 or 3 by about 34 Å or less. This model would place the distance in question at less than 34 Å. While it is not yet possible to determine uniquely a donor–acceptor distance in any given system, the Dale–Eisinger approach when combined with available structural information and or hydrodynamic data can provide a reasonable estimate of the distance. In the present study the energy-transfer results are interpreted on this basis, and the derived donor–acceptor separation can be regarded as a realistic value. The present results also indicate that in the absence of other types of information the values of $R_0({}^2/_3)$ and $R({}^2/_3)$ must be accepted with caution. Similar methodologies should be used to evaluate molecular distances in contractile proteins.

In attempting to evaluate the possible range of R from a different approach, we considered the statistical interpretation of energy-transfer data based on a probability density function of κ^2 (Jones, 1970; Hillel & Wu, 1976; Stryer, 1978). While this approach led to a slightly narrower range of R with the lower limit also being 29 Å, the recent study of Dale et al. (1979) has raised some doubt as to the applicability of such a method to real situations. For this reason, our present results are discussed in terms of the dynamic averaging method.

Similar considerations led to 26.0–50.9 and 25.8–51.3 Å as the best estimated ranges of R for the half-saturated and fully saturated Ca^{2+} complexes, respectively. As with the apoprotein, the $R({}^2/_3)$ for these samples are likely to be in error by more than 20%. Although the minimum distance is 2.5 Å shorter in the Ca^{2+} complexes than in Ca^{2+}-free TNC, R(max) and $R({}^2/_3)$ are unchanged. For reasons already indicated, it is not possible to ascertain what the actual value of R is within each of the estimated ranges. The present results suggest that the effect of Ca^{2+} binding on R is either negligible or a very small decrease in the minimum donor–acceptor separation. The intramolecular distance of TNC from this

Table IV: Parameters for Energy Transfer between DNZ and IAF Attached to TNC[a]

	$-Ca^{2+}$	$+Ca^{2+}$
E	0.66	0.78
$J \times 10^{-15}$ (M^{-1} cm^{-1} nm^4)	0.90	1.1
$R_0(^2/_3)$ (Å)	36.9	41.9
$R(^2/_3)$ (Å)	33.1	34.0
R(min) (Å)	22.8	18.2
R(max) (Å)	42.5	39.3

[a] DNZ was attached to Met-25 and IAF to Cys-98. When Ca^{2+} was present ($+Ca^{2+}$), all four sites were saturated (2.0 mM EGTA plus 2.5 mM Ca^{2+}). Protein concentration was 10 μM.

work forms the basis for future studies of intramolecular distances of TNT and TNI and intermolecular distances among the three subunits of troponin. These distances will allow estimate of their geometric relationship in whole troponin and the effect of cation binding on this relationship.

Energy-Transfer Studies of TNC-DNZ-IAF. In our initial study, we used IAF as an acceptor. It was difficult to separate the IAF emission from the DNZ fluorescence in the donor–acceptor-labeled protein because of the extensive overlap between their fluorescence emisson spectra (TNC-IAF emits maximally near 525 nm and TNC-DNZ in the region 522–512 nm). Nevertheless, we were able to determine the transfer efficiency at wavelengths below 470 nm since the IAF emission spectrum drops off more rapidly than the DNZ spectrum on the short wavelength side. Several results obtained from the DNZ-IAF pair are given in Table IV.

Qualitatively, the results from the DNZ-IAF pair are in agreement with those from DNZ-IAE. Ca^{2+} had little or no effect on $R(^2/_3)$, although the transfer efficiency was larger in the Ca^{2+} complex. The values of $R(^2/_3)$ are 5 Å smaller in the DNZ-IAF conjugates than in the DNZ-IAE proteins. Our measurements on TNC labeled with DNZ and IAF (TNC-DNZ-IAF) were not as detailed as on the DNZ-IAE conjugate. Since the depolarization factor for TNC-IAF was not determined, the estimate on the lower and upper bounds for κ^2 was less accurate than those for TNC-DNZ-IAE. We estimated from Dale et al. (1979) that κ^2(min) was 0.068 and κ^2(max) was 3.45 for Ca^{2+}-free TNC, and κ^2(min) was 0.030 and κ^2(max) was 3.73 for the fully saturated Ca^{2+} complex. These parameters correspond to the distance range 22.8–42.5 Å for Ca^{2+}-free TNC and 18.2–39.3 Å for the Ca^{2+} complex. As with $R(^2/_3)$, R(min) is shorter in this system than in the DNZ-IAE protein. The error in $R(^2/_3)$ is also estimated to be greater than 20%, based on the estimate limits of κ^2.

Since the values of κ^2(min) and κ^2(max) were estimated without knowledge of acceptor depolarization, the actual value for κ^2(min) is likely to be larger than 0.068 and hence R(min) can be expected to be larger than 23 Å for Ca^{2+}-free TNC and 18 Å for the Ca^{2+} complex. Thus, R(min) determined from both IAE and IAF acceptors may be closer to each other than the present results suggest. On the other hand, acceptor depolarization data from TNC-IAF may lead to a decrease in R(max). When the results from both acceptors are taken together, the minimum distance between the donor and acceptor sites can be placed in the range 23–29 Å. Ca^{2+} binding may result in a small decrease to 18–26 Å. Since the lower limits of these ranges are likely to be in error due to the lack of information on IAF depolarization, the minimum separations are likely to be larger than the lower bounds indicated by the IAF results.

Transfer of Structural Information across TNC. Several lines of evidence suggest that Ca^{2+} binding to the high-affinity sites induces large-scale conformational changes in the C-terminal half of the molecule (Levine et al., 1977; Nagy & Gergely, 1979). If these changes resulted in a significant alteration in the spatial position of the attached acceptor relative to the donor, this alteration would be reflected in the measured separation of the two groups. The present results do not suggest such a structural perturbation. While the lack of an observed Ca^{2+} effect on R may not in itself be a strong argument for the absence of global change, the result when combined with observed rotational correlation times is evidence that Ca^{2+} does not induce a significant change in the overall shape of the molecule.

It is of interest to examine over how long a distance information on structural changes may be transmitted from the high-affinity sites to the attached DNZ. When TNC-DNC is titrated with Ca^{2+} at the high-affinity sites, the probe at a distant site responds spectrally to the binding as reflected not only by a small reduction in quantum yield and a red spectral shift but also by a decrease in its orientational freedom (Table III). Since it is generally assumed that the high-affinity sites 3 and 4 (residues 139–150) are equivalent, it is difficult to determine whether the initial DNZ spectral changes which occur during Ca^{2+} titration reflect structural perturbations induced by Ca^{2+} at a single site or both sites together. The predicted structure of TNC indicates that a peptide segment including site 4 is folded back on to the region of site 3 (residues 103–114). In this model the initial perturbation induced by Ca^{2+} binding to either one or the other high-affinity site would be localized in a region very close to Cys-98. Since the distance between the latter site and Met-25 is not significantly altered by Ca^{2+} binding, in order for DNZ at Met-25 to sense a Ca^{2+} effect a signal would have to be propagated over a distance in excess of 29 Å. To further establish the possibility of such transfer over a long distance, it would be necessary to investigate TNC fragments such as the thrombin TH1 fragment (residues 1–120), which includes site 3 but not site 4.

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Fluorescence Detection of Increased Local Flexibility Induced by Coenzyme A in Succinyl-CoA Synthetase from *Escherichia coli*†

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ABSTRACT: The enzyme succinyl coenzyme A synthetase catalyzes the formation of succinyl coenzyme A (CoA) from succinate, CoA, and a nucleoside triphosphate. The enzyme has been labeled with dansyl chloride in both the α and β subunits to give an almost fully active fluorescent conjugate. Addition of CoA at 1 mM caused the polarization of the conjugate to fall from ~0.275 to ~0.145. Control studies indicated that this result was not due to effects such as dissociation of the protein, release of bound label, or changes in the lifetime of the bound label. The other substrates, ATP·Mg^{2+}, succinate, or ADP·Mg^{2+}, gave a polarization decrease when added to the conjugate, but to a much smaller degree compared to the effect of CoA. For example, the maximum decrease with the other substrates was observed with ADP·Mg^{2+} where the polarization only fell to 0.245 at 5 mM ADP·Mg^{2+}. Comparison with model compounds indicates that the effect of CoA is specific. Analysis of the polarization data gives rotational relaxation times for the conjugate which are smaller than that expected for the protein considered as a sphere, in both the presence and absence of CoA. Perrin–Weber plots in the presence of CoA are concave to the T/η axis. These results suggest that the dansyl label can be covalently bound to a flexible site on succinyl-CoA synthetase and the flexibility is greatly increased on CoA binding. These results are compatible with a model for succinyl-CoA synthetase in which CoA binding induces a conformational change in the active site and help explain previous kinetic observations that the presence of CoA can affect the rates and partial reactions involving the other enzyme substrates.

Succinyl-CoA synthetase (SCS)[1] catalyzes the following reaction:

$$\text{succinate} + \text{CoA} + \text{NTP} \overset{M^{2+}}{\leftrightarrow} \text{succinyl-CoA} + \text{NDP} + P_i$$

in which NDP and NTP represent purine nucleoside di- and triphosphates, respectively. The enzyme contains two different kinds of subunits, α and β, which are present in equimolar quantities (Bridger, 1971; Brownie & Bridger, 1972). A number of functional studies have implied that protein conformational changes play a role in this reaction, but the nature of these changes has not been specified.

It appears that the active site is located at the points of contact between the α and β subunits. Thus, the α subunit contains a histidine residue that is phosphorylated during catalysis (Bridger, 1971; Brownie & Bridger, 1972). A second histidine residue of undetermined subunit location also may be involved in the catalytic reaction (Collier & Nishimura, 1979). Since the isolated α subunit (Pearson & Bridger, 1975a) is capable of being phosphorylated by ATP, it is reasonable to conclude that the nucleoside di- and triphosphate binding site, or part of it, is located on the α subunit (Pearson & Bridger, 1975b). Evidence consistent with participation of both subunits in the composition of this site has been presented recently (Ball & Nishimura, 1980). Indeed, Pearson &

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[1] Abbreviations: CoA, coenzyme A; SCS, succinyl-CoA synthetase; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; o-$CoAS_2$, oxidized CoA disulfide; DSCoA, desulfo-CoA; dansyl, 8-(dimethylamino)-1-naphthalenesulfonate.

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