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STUDIES ON THE INTERACTIONS BETWEEN THE SUBUNITS OF SKELETAL MUSCLE TROPONIN USING FLUORESCENCE QUENCHING, PHOTOCHEMICAL CROSS-LINKING, AND EXCITATION ENERGY TRANSFER TECHNIQUES

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The thin filament proteins troponin (Tn) and tropomyosin are responsible for the regulation of skeletal muscle contraction by Ca^{2+}. The first event in this Ca^{2+}-regulation process is the binding of Ca^{2+} to TnC, one of the three subunits of Tn. To understand how this Ca^{2+}-triggering signal is tramsitted from TnC to the other components of the thin filament, we have investigated the interactions between TnC and the other two Tn subunits, TnI and TnT, using three different but complementary techniques. Using fluorescence quenching techniques, we have previously shown that the Stern-Volmer quenching rate constant for TnC labeled at its single sulfhydryl group (Cys-98) with the fluorescence probe 1,5-IAEDANS[1] decreases by a factor of 3.5 when either TnI or TnT are bound. This is consistent with the view that in both the TnC·TnI and the TnC·TnT binary complexes the TnI or TnT moieties bind to TnC near the probe attachment site and thus shield the probe from collision with quenchers in the medium. In the present studies, the proximity relationships between TnC and TnI or TnT were further explored using photochemical cross-linking and fluorescence energy transfer techniques.

MATERIALS AND METHODS

TnC was labeled at Cys-98 with the site-specific photocrosslinker BP-Mal (Molecular Probes, Junction City, OR) as described (2). Irradiation was carried out in a Rayonet RPR-100 "Photochemical Reactor" equipped with 16 "3,500" lamps (Southern New England Ultraviolet, Hamden CT). TnI and TnT were ^{3}H-labeled before cross-linking by reductive methylation (3). Gel bands were cut out and counted as previously described (4). Labeling of TnC at Cys-98 with 1,5-IAEDANS was carried out as described in reference 5. Labeling of TnI at Cys-133 with DAB-Mal (Molecular Probes) was carried out by incubating intact Tn with a twofold molar excess of DAB-Mal at 25° for 2 h in a buffer containing 0.1 M KCl, 0.1 mM $CaCl_2$, 20 mM Hepes, pH 7.5. After dialysis to remove unreacted reagents, the labeled TnI was isolated by urea-DEAE column chromatography. Steady-state fluorometry was carried out on a Perkin-Elmer MPF-4A spectrofluorometer. Fluorescence lifetime measurements were done on a modified Ortec 9,200 ns fluorometer as previously described (6). All experiments were carried out in a buffer containing 0.1 M KCl, 20 mM Hepes, pH 7.5, at 25°.

RESULTS AND DISCUSSION

Our results show that TnI or TnT can be cross-linked to BP-TnC after irradiation of the BP-TnC·TnI or the BP-TnC·TnT binary complex (Fig. 1), confirming the conclusion derived from our fluorescence quenching studies that either subunit binds to TnC in the vicinity of Cys-98. Irradiation of the BP-TnC·TnI.TnT ternary complex produced multiple cross-linking bands (Fig. 1) whose identities were ascertained by using ^{3}H-labeled TnI or TnT initially to form the complex. These studies established that both TnI and TnT are cross-linked to BP-TnC in the ternary complex, suggesting a geometry for the Tn complex in which all three subunits bind near Cys 98 of TnC.

Both steady-state and lifetime measurements revealed that in the ternary complex, substantial energy transfer

[1] *Abbreviations used in this paper:* 1,5-IAEDANS, 5-[2-(iodoacetyl)aminoethyl] aminonaphthalene-1-sulfonic acid; DAB-Mal 4-dimethylaminophenylazophenyl-4-maleimide; BP-Mal, benzophenone-4-maleimide; BP-TnC, TnC labeled with BP-Mal.

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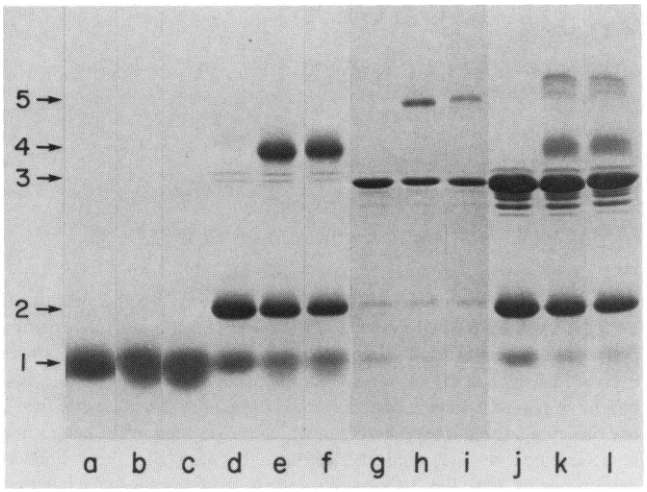

FIGURE 1 Crosslinking of BP-TnC to TnI and TnT. SDS polyacrylamide (14%) gel electrophoresis; BP-TnC alone (lanes *a*, *b*, *c*), BP-TnC·TnI (lanes *d*, *e*, *f*), BP-TnC·TnI (lanes *g*, *h*, *i*), and BP-TnC·TnI.TnT (lanes *j*, *k*, *l*). Lanes *a*, *d*, *g*, *j*, before irradiation; lanes *b*, *e*, *h*, *k*, samples irradiated in the presence of 0.1 mM $CaCl_2$; lanes *c*, *f*, *i*, *l*, samples irradiated in the presence of 1 mM EGTA. Band 1, BP-TnC; band 2, TnI; band 3, TnT; band 4, BP-TnC cross-linked with TnI; band 5; BP-TnC cross-linked with TnT.

occurs between the AEDANS donor attached at Cys-98 of TnC, and the DAB acceptor attached at Cys-133 of TnI. From the measured donor lifetimes in the absence and presence of acceptor, and assuming an orientation factor, $\kappa^2 = 2/3$, we obtained interprobe distances of 3.8 nm for the metal-free complex, 4.0 nm with the high affinity sites occupied by Mg^{2+}, and 3.4 nm with the low affinity sites occupied by Ca^{2+} (Table I). In the absence of Mg^{2+}, Ca^{2+} titration of the change in donor fluorescence in the presence of acceptor yields a biphasic curve (Fig. 2). The initial rise in fluorescence, corresponding to an increase in the

TABLE I
ENERGY TRANSFER PARAMETERS AS A FUNCTION OF METAL IONS PRESENT

	τ_d	τ_{da}	E	R
	ns	*ns*	%	*nm*
$+Ca^{2+}$	17.29	4.83	72	3.40
$+Mg^{2+}$	17.71	8.45	52	3.95
+EGTA	18.00	7.34	59	3.76

Samples are either the AEDANS-TnC.DAB-TnI.TnT or the AEDANS-TnC.TnI.TnT ternary complexes. τ_d and τ_{da} are donor lifetimes in the absence and presence of acceptor, respectively. *E* is the energy transfer efficiency. *R* is the separation distance calculated from *E* and a critical transfer distance (R_0) of 4.0 nm.

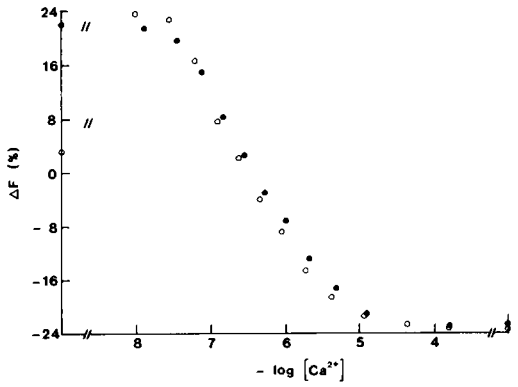

FIGURE 2 Ca^{2+}-titration of fractional changes in fluorescence (ΔF) for the ternary AEDANS-TnC.DAB-TnI.TnT complex. ○ are in the absence of Mg^{2+}, ● are in the presence of 2 mM $MgCl_2$. Ca^{2+} concentrations were adjusted using a Ca^{2+}-EGTA buffer system.

interprobe distance when Ca^{2+} binds first to the high affinity sites, is followed by a decrease when the lower affinity sites are occupied ($pCa_{1/2} \sim 7.5$). Because the low affinity sites have been implicated in the triggering of the activation of actomyosin ATPase, it seems that a key event in the Ca^{2+}-regulation process is a 0.6 nm movement of the Cys-133 region in TnI toward the Cys-98 region in TnC.

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