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CIRCULAR DICHROISM AND FLUORESCENCE STUDIES ON TROPONIN–TROPOMYOSIN INTERACTIONS

R. S. MANI, W. D. McCUBBIN and C. M. KAY

Medical Research Council Group on Protein Structure and Function and the Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

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1. Introduction

The regulatory apparatus for vertebrate striated muscle contraction is located at the thin filaments and consists of troponin (a complex of three separate subunits) in interaction with tropomyosin and actin [1–3]. It has recently been shown that troponin, tropomyosin and actin are present in a 1:1:7 molar ratio in myofibrils [4]. In 1963, Lowy and Hanson suggested that tropomyosin particles were longitudinally arranged in the two long pitch grooves of the F actin double helix [5], and this has presently been confirmed by several laboratories utilizing X-ray and electron diffraction techniques [6–8]. These studies also demonstrated that the precise location of tropomyosin on the actin helix depends on the state of troponin and the level of calcium ions present. In the relaxed state, i.e., at low levels of calcium, tropomyosin molecules are in such a position as to prevent the interaction of the myosin 'heads' with actin. The binding of calcium ions by troponin C produces a dramatic conformational change in this molecule [9] which apparently is relayed via troponin T to tropomyosin, and this entity moves nearer the middle of the actin groove, thereby permitting the interaction of myosin with actin. On the basis of these findings and their own data, Potter and Gergely [10] have presented a model for the regulatory mechanism. An interesting feature of this model is the suggestion that an induced conformational change in tropomyosin favors its movement to the centre of the groove upon activation. In this communication results from circular dichroism, ellipticity melting experiments and fluorescence studies are presented, which were designed to explore possible conformational changes induced in tropomyosin upon interaction with other members of the troponin complex in the presence of calcium ion.

**Abbreviations*: CD, circular dichroism, CM, carboxymethyl; DEAE, diethylamino ethyl; EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N'* tetraacetic acid; TN-C, calcium binding subunit of troponin, TN-T, tropomyosin binding subunit; TM_B, tropomyosin; TN-CT, protein complex of TN-C and TN-T; TN-T-TM_B, protein complex of TN-T and TM_B; TN-CT-TM_B, protein complex of TN-C, TN-T and TM_B: T_m, melting temperature; R_{tryp}, relative fluorescence intensity.

2. Materials and methods

2.1. *Protein isolation and purification*

Troponin was extracted from rabbit skeletal muscle employing a slight modification of the procedure of Ebashi et al. [11], and separated into its components by chromatography on DEAE Sephadex and CM cellulose [12,13]. Tropomyosin was extracted from the residue used for the troponin preparation and was purified by successive cycles of isoelectric precipitation and ammonium sulfate fractionation. All proteins used in these studies were homogeneous by the criterion of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [14].

2.2. *Protein concentration*

Troponin protein concentrations were routinely measured by ultraviolet absorption employing previously established extinction coefficients [15]. For TM_B, a

value of $E^{1\%}_{1\,cm,\,278\,nm}$ of 3.45 was used. The various protein complexes studied were formed by mixing the individual components always in equimolar ratios. Prior to mixing, the proteins were separately dialyzed against 0.5 M KCl, 1 mM EGTA, 50 mM Tris–HCl buffer at pH 8.0.

2.3. *Circular dichroism*

The CD measurements and ellipticity melting experiments were made on a Cary Model 6001 circular dichroism attachment to a Cary 60 recording spectropolarimeter according to previously described methodology [16,17].

2.4. *Fluorescence studies*

These were carried out in a Turner Model 210 recording spectrofluorometer, with water jacketed sample compartment and a constant temperature of 20°C was maintained by a Lauda thermoregulator. Tryptophan fluorescence of the various complexes was observed at 350–355 nm, after excitation at 295 nm. The relative fluorescence (R_{tryp}) was determined by comparing the fluorescence intensity of the sample at the emission peak with that of tryptophan using the relationship:

$$R_{tryp} = \frac{\text{fluorescence intensity of sample}}{\text{fluorescence intensity of tryptophan}}$$

$$\frac{OD_{tryp}\ 295\ nm}{OD_{sample}\ 295\ nm}$$

2.5. *Ca^{2+} concentrations*

The available or free calcium concentrations were adjusted by means of a Ca^{2+} buffer consisting of Ca^{2+} and 1 mM EGTA in 0.5 M KCl, 50 mM Tris–HCl buffer at pH 8.0 employing a binding constant for EGTA of $2 \times 10^7\ M^{-1}$ [15].

3. Results and discussion

3.1. *Circular dichroism*

Far ultraviolet CD spectra of TN-T, TM_B and TN-T-TM_B were recorded in the solvent system 0.5 M KCl, 1 mM EGTA, 50 mM Tris–HCl at pH 8.0. The results are presented in fig.1. Also included in this figure is a calculated spectrum of the complex TN-T-TM_B obtained by taking a molar contribution from each species and assuming no interprotein interactions. The values of $[\theta]_{221\,nm}$ of −36 800 deg $cm^2\ dmol^{-1}$ and −8000 deg $cm^2\ dmol^{-1}$ for TM_B and TN-T, respectively, are essentially identical with previously published values in different solvent systems [16,18]. All the experimental spectra were unaltered by the addition of Ca^{2+} ion. This is a rather important observation because it has been suggested that the conformation of TM_B may be altered by the presence of Ca^{2+}, thus permitting movement in the actin groove upon activation [19]. The CD results, however, do not preclude the possibility of very subtle changes occurring in TM_B in vivo.

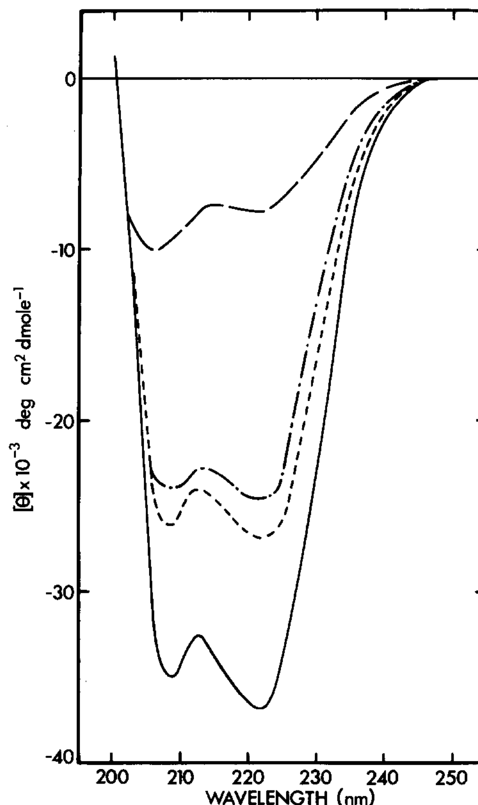

Fig.1. Far ultraviolet circular dichroism spectra of TN-T (—–), TM_B (——), TN-T-TM_B (. – .) and the theoretical circular dichroism spectrum calculated for TN-T-TM_B (- - - -) in 0.5 M KCl, 50 mM Tris–HCl, 1 mM EGTA (pH 8.0).

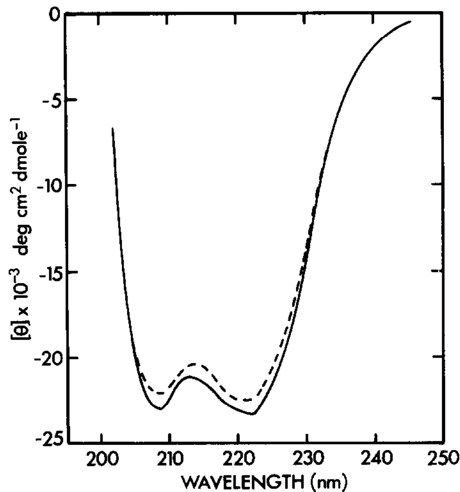

Fig.2. Far ultraviolet circular dichroism spectra of TN-CT-TM_B protein complex in 0.5 M KCl, 50 mM Tris–HCl, 1 mM EGTA (pH 8.0) (- - - -) and in 0.5 M KCl, 50 mM Tris–HCl, 1 mM EGTA, 5×10^{-4} free Ca^{2+} at pH 8.0 (——).

The experimental value of $[\theta]_{221\ nm}$ for TN-T-TM_B of $-24\ 300 \pm 300$ deg cm^2 $dmol^{-1}$ is to be compared with the theoretical value of $-26\ 800 \pm 300$ deg cm^{-2} $dmol^{-1}$. This ellipticity loss of 2500°, or 10 percent, is quite significant and obviously reflects a fairly substantial conformational alteration. The suggestion made here is that the ellipticity loss reflects a conformational change in the TM_B moiety, induced by complexation with TN-T. It would seem very unlikely that the already small amount of secondary structure present in TN-T would be reduced a further 50% by interaction with TM_B.

Previous observations from this laboratory have developed the idea of the Ca^{2+} induced change in TN-C being intimately involved as the biological trigger which controls muscle contraction [9,15,17,20]. It was a logical extension of these studies to include the contribution of TM_B to a complex with TN-CT. Fig.2 displays the far UV CD spectra of the complex TN-CT-TM_B in the absence and presence of Ca^{2+}. It is apparent that the Ca^{2+} induced change in TN-C, which has already been demonstrated to occur in the complex TN-CT [15] is still operative when the TN-T moiety complexes with TM_B. The values of $[\theta]_{221\ nm}$ of $-22\ 300 \pm 300$ deg cm^2 $dmol^{-1}$ and $-23\ 200 \pm 300$ deg cm^2 $dmol^{-1}$ for the 'minus' and 'plus' Ca^{2+} states are compared with the theoretical values of $-24\ 200 \pm 300$ deg cm^2 $dmol^{-1}$ and $-24\ 900 \pm 300$ deg cm^2 $dmol^{-1}$, respectively. These values also suggest that no further change is produced in TM_B by the presence of TN-C attached to TN-T, when the complex is formed.

3.2. *Ellipticity melt experiments*

The thermal stability of TM_B and complexes with TN-T and TN-C was studied by carrying out ellipticity melting experiments in 0.5 M KCl, 1 mM EGTA, 50 mM Tris–HCl at pH 8.0 and in the same solvent containing Ca^{2+}. Fig.3 presents data for TM_B and the complex TN-T-TM_B. From the derivative plots of these $[\theta]_{221\ nm}$ versus T°C graphs (inserts), it is possible to measure actual melting temperatures. For TM_B there are 2 transitions at: 43–44°C and 51–52°C and for the complex, TN-T-TM_B, only one at 44–45°C. These values were found to be unaffected by Ca^{2+}. TN-T does not yield an abrupt T_m but shows a progressive loss of ellipticity [15].

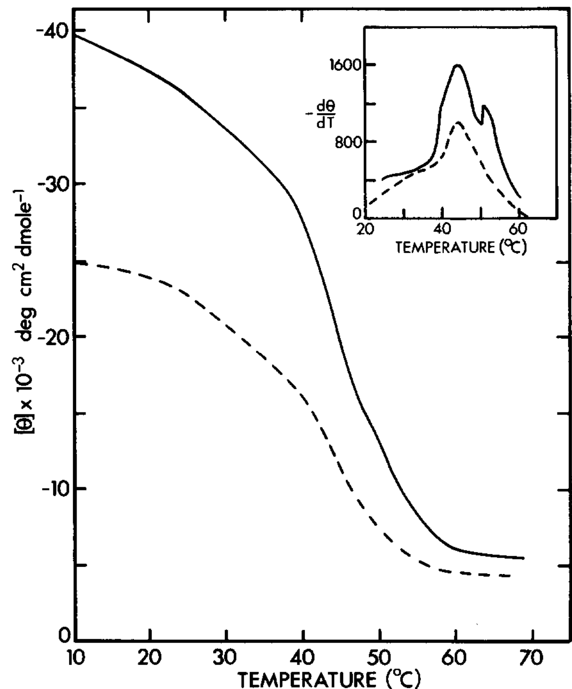

Fig.3. The change in ellipticity at 221 nm as a function of temperature for TM_B (——) and TN-T-TM_B (- - - -) in 0.5 M KCl, 50 mM Tris–HCl, 1 mM EGTA (pH 8.0). Inserts: derivative plots with respect to temperature.

Increased thermal stability of TN-CT in the presence of Ca^{2+} has already been noted [15], and as the data in fig.4 demonstrate, this is also the case for the complex with TM_B. A T_m value of 43–44°C was observed in the absence of Ca^{2+} while two transitions with T_m values of 43 and 53°C were computed in the presence of Ca^{2+}. These observations are explained as follows: TM_B is highly α helical, in excess of 92% at room temperature, and thus its melting behavior would be expected to be of a co-operative nature [21]. The data in fig.3 reveals that the helical structure is not of uniform stability since complete unfolding of the molecule occurs through two co-operative transitions with T_m values of 43 and 52°C. In this regard, extensive fluorescence studies [22] have shown that tropomyosin undergoes a conformational change at 34°C into an intermediate state between rigid rod and completely unfolded chain. It seems important that this intermediate conformation is stable in a temperature range from 34 to 43°C, since this includes physiological temperature. In the complex, TN-T-TM_B, it is suggested that some disorder is produced in the

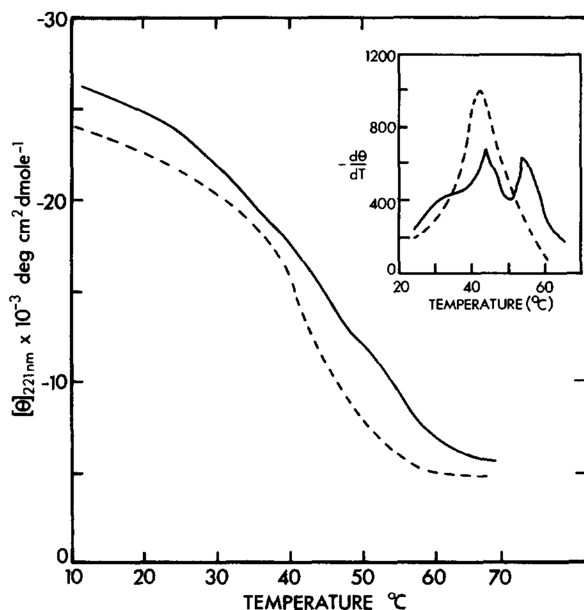

Fig.4. The change in ellipticity at 221 nm as a function of temperature for TN-CT-TM_B in 0.5 M KCl, 50 mM Tris–HCl, 1 mM EGTA (pH 8.0) (- - - -) and in 0.5 M KCl, 50 mM Tris–HCl, 1 mM EGTA, 5×10^{-4} M free Ca^{2+} at pH 8.0 (——). Inserts: derivative plots with respect to temperature.

TM_B moiety, perhaps akin to the thermal activation. Thus one might expect that the higher T_m (53°C) characteristic of the α helix melting would be repressed and the monophasic melting profile would only show the lower T_m of 43°C. This argument could also apply in the case of TN-CT-TM_B in the absence of Ca^{2+}, where again one finds the single lower T_m of 44°C. However, in the presence of Ca^{2+}, more order is generated by virtue of a tighter TN-CT interaction and TM_B reverts more to its initial state characterized by two T_m values of 43 and 51°C.

3.3. *Fluorescence studies*

TM_B and TN-C are devoid of tryptophan and hence will have essentially no intrinsic fluorescence when excited at 295 nm. TN-T has two tryptophan residues per mol, the fluorescence of which will be sensitive to any change in the environment of these groups upon complex formation. Table 1 summarizes the fluorescence results that were obtained. For TN-T alone the emission maximum occurs at 353–355 nm, a value close to that noted for indole in water. This suggests that in TN-T the 2 tryptophan residues are freely exposed to the aqueous solvent. The lack of any significant blue shift of the emission maximum in TN-T-TM_B precludes any burying of the tryptophans when TM_B binds; however, the increase in the R_{tryp} value from 0.85 to 1.00 suggests that the chromophores are sensing a change in the microenvironment. The R_{tryp} values of 0.85 and 1.00 for TN-T and TN-T-TM_B are intermediate between values for tryptophans in randomly coiled polypeptides and for indoles exposed to an aqueous environment, but lacking carbonyl substituents [23]. For TN-CT, a slight quenching occurs which is restored by the presence of TM_B. Ca^{2+}

Table 1
Intrinsic tryptophan fluorescence of TN-T and its complexes with TN-C and TM_B

Sample	Emission maximum, nm	R_{tryp}
TN-T	353–355	0.85
TN-T-TM_B	351–353	1.00
TN-CT	353–355	0.75
TN-CT-TM_B	351–353	0.95

Solvent system was 0.5 M KCl, 1 mM EGTA 50 mM Tris–HCl at pH 8.0. Temperature, 20°C.

had no significant effect on either the emission maximum or R_{tryp} in any of these systems.

In conclusion, the results of this investigation suggest a conformational change in TM_B upon complex formation with TN-T. Although it was not possible to demonstrate a direct effect by Ca^{2+} on TM_B alone, it is conceivable that in vivo, the binding of Ca^{2+} by TN-C, with the consequent conformational change in this molecule, influences the modified form of TM_B via the attached TN-T, thereby producing the release of the inhibitory protein anchor with consequent movement of TM_B in the actin groove.

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