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Coupled Responses of the Regions Near Cysteine-190 and the Carboxy Terminus of Rabbit Cardiac Tropomyosin: Fluorescence and Circular Dichroism Studies†

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ABSTRACT: Rabbit cardiac tropomyosin was labeled at Cys-190 with either *N*-(1-pyrenyl)iodoacetamide (Py) or 6-acryloyl-2-(dimethylamino)naphthalene (AD, acrylodan). Half of the labeled sample then was treated with carboxypeptidase A to produce an identically labeled nonpolymerizable form of tropomyosin, NPTM. Investigation of temperature-dependent changes in pyrene excimer emission, acrylodan fluorescence polarization, and tyrosyl circular dichroism in different samples of tropomyosin and NPTM reveals that absence of the COOH-terminal portion of tropomyosin modifies the response of the Cys-190 region to heat. Removal of the COOH terminus releases certain conformational constraints from the coiled-coil back to and including the Cys-190 region without causing a severe drop in the net α-helical content of the protein. Observation of changes in pyrene excimer fluorescence and in fluorescence polarization of acrylodan with time after addition of carboxypeptidase A to samples of labeled tropomyosin directly demonstrates this relaxation process. Thermally induced reduction in tyrosyl circular dichroism, together with consideration of the distribution of tyrosyl residues on tropomyosin, also supports the proposal.

Tropomyosin (TM)[1] is a rodlike molecule composed of two parallel α-helical polypeptide chains wrapped around each other to form a coiled coil [reviewed by Smillie (1979), Côté (1983), and Marston and Smith (1985)]. TM from rabbit cardiac muscle consists of 2 identical chains, each 284 residues long and each containing a single cysteine residue at position 190 (Lewis & Smillie, 1980). In skeletal and cardiac muscles, TM lies in a head-to-tail manner along the grooves of F-actin filaments and works with troponin to confer Ca^{2+} sensitivity to muscle contraction [reviewed by McCubbin and Kay (1980)]. The end-to-end interactions of TM are linked to its function in the Ca^{2+}-dependent regulatory systems of striated muscle. They involve the overlap of eight or nine amino acids (Johnson & Smillie, 1975, 1977; Mclachlan & Stewart, 1975; Phillips et al., 1986) and are manifested in low ionic strength solutions in vitro by an increased solution viscosity relative to solutions at physiological or higher ionic strengths. This increase in viscosity can be abolished by removal of COOH-terminal amino acids by treatment with carboxypeptidase A to produce a nonpolymerizable form of TM (NPTM) (Ueno et al., 1976; Mak & Smillie, 1981a). NPTM alone does not bind to F-actin but can be induced to do so by the addition of whole troponin in the presence or absence of Ca^{2+} (Heeley et al., 1987). This ternary system in the absence of free Ca^{2+} produces myosin S1 ATPase inhibition comparable to that of a system with TM instead of NPTM, but activation of S1 ATPase activity by addition of Ca^{2+} is much reduced in the NPTM-containing system (Heeley et al., 1989a).

TM fully dephosphorylated at Ser-283 exhibits a lower degree of polymerizability than fully phosphorylated TM (Heeley et al., 1989b). Polymerizability can also be disrupted by changes at positions well removed from the COOH terminus, e.g., interaction with deoxyribonuclease I (Payne et al., 1986; Clark & Burtnick, 1989) and chemical modification at Cys-190 (Graceffa & Lehrer, 1980; Burtnick & Racic, 1988). ^{1}H NMR studies of His-153 and His-276 of rabbit cardiac tropomyosin (Edwards & Sykes, 1980) suggest there to be a number of intermediate folded structures along the pathway of thermal denaturation for tropomyosin. Consistent with the idea of long-range communication along the tropomyosin coiled coil, carboxymethylation or cystine formation at Cys-190 alters the thermal dependence of the NMR signals from these His residues some 5.5 and 12.8 nm away, respectively.

In this study, we have attempted to increase our understanding of how events that occur near Cys-190 are communicated to the COOH terminus and vice versa by comparing fluorescence and circular dichroism (CD) responses of intact and carboxypeptidase-truncated TM molecules. One of the

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[1] Abbreviations: Py, *N*-(1-pyrenyl)iodoacetamide; acrylodan (AD), 6-acryloyl-2-(dimethylamino)naphthalene; TM, tropomyosin; NPTM, nonpolymerizable rabbit cardiac tropomyosin prepared by digestion with carboxypeptidase A; DTT, DL-dithiothreitol; Mops, 3-(*N*-morpholino)-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl.

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fluorescent probes used was *N*-(1-pyrenyl)iodoacetamide (Py). The sites of attachment of pyrene at Cys-190 residues on adjacent TM chains allow the possibility of pyrene excimer formation (Betcher-Lange & Lehrer, 1978). Graceffa and Lehrer (1980) contend, in a model later expanded by Ishii and Lehrer (1985), that TM labeled with *N*-(1-pyrenyl)maleimide equilibrates between two different conformations. One is proposed to be a rigid coiled-coil with its attached pyrenes unable to stack sufficiently well to form an excimer. The second is a less constrained coiled coil, tentatively associated with localized unfolding of the chains, in which excimer formation could be achieved. Pyrene-based labels on TM have been used widely to study conformational changes at the probe site (Lin, 1982; Ishii & Lehrer, 1985, 1990; Burtnick et al., 1986, 1988) in response to physical perturbations of the sample or to interaction with other proteins.

The second fluorescent probe used was 6-acryloyl-2-(dimethylamino)naphthalene (acrylodan, AD). Acrylodan exhibits a high degree of fluorescence polarization when bound to TM (Clark & Burtnick, 1988; Lehrer & Ishii, 1988). Changes in acrylodan fluorescence polarization provide information complementary to that from the Py studies.

In addition, as the tyrosine residues of TM are concentrated in its COOH-terminal third, tyrosine CD can be used to monitor conformation changes in that part of TM (Bullard et al., 1976; Nagy, 1977; Holtzer et al., 1989). Cys-190 on TM is about one-third of the length of the molecule in from its COOH terminus. Results of the studies with fluorescent labels can be discussed in light of CD data both of tyrosine ring, and of peptide bond origins.

Materials and Methods

Proteins. Rabbit cardiac TM and NPTM were prepared according to Mak and Smillie (1981a). Cardiac TM was dephosphorylated with *Escherichia coli* alkaline phosphatase (Pharmacia) according to Heeley et al. (1987) prior to treatment with carboxypeptidase A (Sigma type I, treated by the supplier with diisopropyl fluorophosphate and by ourselves with phenylmethanesulfonyl fluoride to eliminate tryptic and chymotryptic activities). After treatment with and subsequent removal of carboxypeptidase A by heating to 80 °C and centrifugation at 15000*g* for 10 min, a 20-μL aliquot of the NPTM solution was reacted with 20 μL of 10 mM dansyl chloride in acetone and subjected to TLC on polyamide sheets (Woods & Wang, 1967). The dansylamino acids then were identified by comparison of their mobilities in different solvent systems with those of standard dansylamino acids (Sigma) (Perham, 1978). In agreement with amino acid analysis of the products (Mak & Smillie, 1981a), we found that 9–11 residues were removed by carboxypeptidase A. Labeled TM samples were treated similarly with carboxypeptidase A to produce labeled NPTM species. Analyses of the amino acids released by carboxypeptidase A digestion of Py-labeled and AD-labeled tropomyosins produced thin-layer chromatograms that were indistinguishable from those of digests of unlabeled TM. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970) of the products revealed single bands by fluorescence analysis both prior to staining and subsequent to staining with Coomassie Blue. To ensure identical degrees of labeling of TM and NPTM in a given experiment, the NPTM was prepared directly from the same stock solution of labeled intact TM against which it was to be compared. This is an important factor in the Py experiments as excimer formation is a function of the degree of labeling.

Concentrations of unlabeled TM and NPTM in solutions were determined spectrophotometrically by using an absorption coefficient of 21 780 M^{-1} cm^{-1} at 277 nm (McCubbin & Kay, 1969), with the assumption that the absorption coefficient would remain unchanged after treatment with carboxypeptidase A as no tyrosine residues are among those removed.

Labeling of TM. To prepare TM fluorescently labeled at Cys-190, dephosphorylated TM (2 mg/mL) initially was dialyzed overnight against 150 mM KCl, 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and 5 mM dithiothreitol (DTT), pH 8.0, at 4.0 °C. The TM then was dialyzed for 4–5 h against 150 mM KCl/10 mM Tris-HCl, pH 8.0 (no DTT), after which either Py or AD (Molecular Probes), dissolved in a minimal volume of *N*,*N*-dimethylformamide, was added to an approximate 10-fold molar excess over TM. The reaction was allowed to proceed for 2–4 h at 37 °C in the dark. The reaction mixture then was centrifuged at 15000*g* for 10 min to remove precipitated reagent and the supernatant dialyzed overnight against 150 mM KCl/20 mM Mops, pH 7.0. Residual unreacted reagent was removed by gel filtration through Bio-Gel P2 (Bio-Rad).

The extent of labeling of TM with Py or AD was quantified by independent determinations of the amounts of label and protein in a sample solution. Concentrations of fluorescent labels were determined by using molar absorption coefficients of 2.2×10^4 M^{-1} cm^{-1} at 344 nm for Py (Kouyama & Mihashi, 1981) and 1.29×10^4 M^{-1} cm^{-1} at 360 nm for AD (Prendergast et al., 1983). Protein concentrations of labeled samples were determined by using the method of Bradford (1976) as adapted for use with the Bio-Rad protein staining reagent.

Optical Methods. Steady-state fluorescence spectra were collected by using a Perkin-Elmer LS 5B luminescence spectrometer connected to a Perkin-Elmer 7500 computer. Fluorescence polarization values, $p = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, were determined by using PTPOL software (Perkin-Elmer) and a polarization accessory for the LS 5B fluorometer. $I_{\parallel}$ and $I_{\perp}$, respectively, are fluorescence intensities measured parallel and perpendicular to the plane of polarization of the excitation beam, after appropriately taking into account the grating correction factor for the instrument. Temperature control was achieved with a circulating water bath (Haake).

Absorbance measurements were made with a Perkin-Elmer λ 4B spectrophotometer. CD data were collected by using a modified Jasco J-20 spectropolarimeter (Landis Instrument) thermally regulated with a circulating water bath (Grant).

Results and Discussion

Py–TM and Py–NPTM. Degrees of labeling of TM with Py were typically 1.6–2.0 Py's per molecule. Figure 1 shows the effects of temperature on fluorescence intensities from pyrene monomers (measured at 383 nm) and pyrene excimers (measured at 490 nm) in samples of Py–TM and Py–NPTM at approximately physiological ionic strength [Figure 1a, 150 mM KCl/20 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), pH 7.0] and at low ionic strength (Figure 1b, 20 mM Mops, pH 7.0).

Several points should be noted in Figure 1. First, pyrene monomer emission decreases relatively steeply and monotonically for Py–TM and Py–NPTM regardless of the ionic strength of the solution. Such behavior is largely the result of increased rates of dissipation of energy from excited-state chromophores as a result of increased frequency of collision with solvent molecules or increased vibrational mode activity.

Second, excimer fluorescence levels change with temperature in a more complicated way that reflects not only the direct thermal effects on the chromophore but also the indirect effects through thermally induced changes in the structure of the polypeptide chains to which the chromophores are bound.

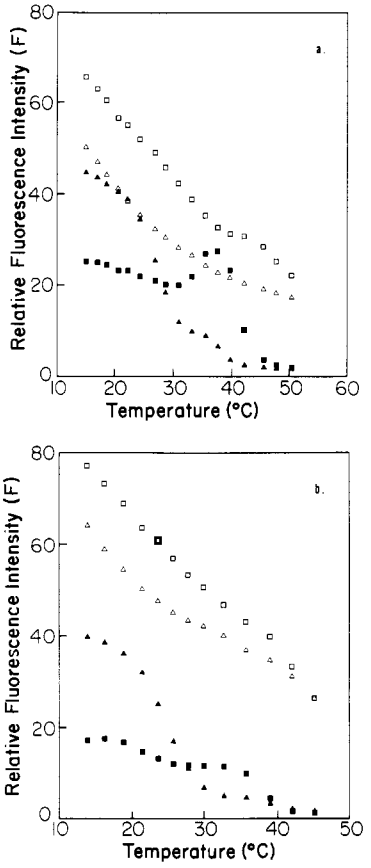

FIGURE 1: Thermal effects on fluorescence of monomers (open symbols) and excimers (closed symbols) in samples of Py–TM (squares) and Py–NPTM (triangles). One micromolar samples of Py–TM and Py–NPTM were heated, and fluorescence spectra were recorded at various temperatures. Monomer and excimer fluorescence intensities were determined, respectively, at 383 and 490 nm at each temperature. Conditions were either (a) 150 mM KCl/20 mM Mops, pH 7.0, or (b) 20 mM Mops, pH 7.0. Excitation was at 280 nm.

Most strikingly, excimer fluorescence intensities from Py–TM in physiological ionic strength solutions decrease slowly as the temperature is raised from 15 °C through about 33 °C but then increase markedly to a maximum near 38 °C. Then they fall again as temperature increases. Similar behavior has been reported recently for rabbit skeletal TM labeled with Py (Ishii & Lehrer, 1990) and interpreted in terms of partial unfolding of the Cys-190 region near 38 °C, which reduces constraints placed by the coiled-coil structure on the approach of an excited-state pyrene at Cys-190 to a neighboring ground-state pyrene at Cys-190 on the second TM chain. Complete separation of the two chains at higher temperatures abolishes excimer formation [also see Ishii & Lehrer (1985)]. The relative instability of the Cys-190 region of TM in solutions of moderate to high ionic strength is well documented (Woods, 1977; Edwards & Sykes, 1980; Williams & Swenson, 1981; Betteridge & Lehrer, 1983; Graceffa & Lehrer, 1984). The absence of a distinct maximum in excimer fluorescence near 38 °C in Py–TM at low ionic strength is consistent with the absence of a pretransition in CD studies of thermal denaturation of TM in low ionic strength solutions (Betteridge & Lehrer, 1983).

Third, Py–NPTM produces changes in excimer fluorescence with temperature that are completely different than those for intact Py–TM. At low temperatures, the excimer emission is much higher in Py–TM, indicative, in terms of the Graceffa and Lehrer (1980) proposal, of a higher proportion of the more open structural form of the coiled coil near Cys-190 for Py–NPTM than for Py–TM. As temperature increases, the fluorescence drops off steeply near 25 °C, suggestive of chain separation in that region at a much lower temperature than in Py–TM. The fact that the only difference between the two molecules is at the COOH terminus suggests that the structure at the COOH terminus acts to hold the Cys-190 region in a specific conformation that responds in a specific way to increases in temperature. On removal of the COOH terminus, the Cys-190 region assumes a more open conformation that is less stable to increases in temperature than in intact Py–TM.

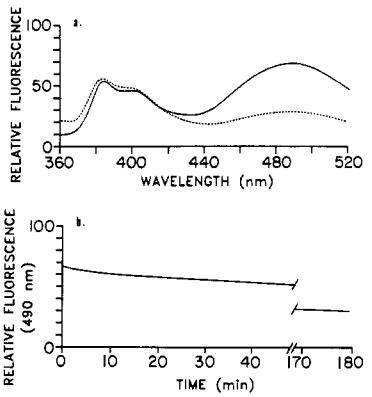

FIGURE 2: (a) Fluorescence spectra of a 1 μM Py–TM sample in 150 mM KCl/20 mM Mops, pH 7.0, recorded before (—) and after (---) 3 h of digestion with carboxypeptidase A at a 1/50 (w/w) ratio to TM. Temperature = 37 °C. Excitation was at 280 nm. (b) Decrease of excimer fluorescence on digestion of a sample from (a) with carboxypeptidase A.

Fourth, in low ionic strength solutions near room temperature, excimer fluorescence levels are lower and monomer fluorescence levels are higher than observed at higher ionic strengths. This may be caused by excimer formation being, in part, entropically driven and, so, being more favorable at high ionic strengths.

Finally, fluorescence emission spectra observed for Py–TM and Py–NPTM in the higher ionic strength buffers, which provided data for Figure 1a, could be changed completely and reversibly to spectra characteristic of those labeled species in low ionic strength buffers by dialysis.

When the above experiment was performed on a sample of Py–NPTM that had not been dephosphorylated prior to treatment with carboxypeptidase A, there was a small but significant peak in the excimer emission profile near 38 °C (results not shown). This was due to the presence of some undigested Py–TM, the result of the inability of carboxypeptidase A to remove residues beyond and including phosphorylated Ser-283, which occurs normally in TM preparations at levels of approximately 20% (Mak et al., 1978). Therefore, all data presented in this paper were obtained with TM that had been dephosphorylated (Heeley et al., 1987). We cannot eliminate the possibility that the small shoulder evident near 38 °C in the excimer emission profile for Py–NPTM is the result of incomplete digestion of TM by carboxypeptidase A.

An alternative representation of the difference in fluorescence properties of Py–TM and Py–NPTM at 37 °C in solutions of physiological ionic strength is provided in Figure 2. The emission spectrum of a sample of Py–TM was recorded (Figure 2a), and then an aliquot of carboxypeptidase A was added. The change in fluorescence at 490 nm (F_{490}) was monitored with time (Figure 2b). After 3 h, the spectrum was recorded again (Figure 2a). The data illustrate the change in conformation at Cys-190 as carboxypeptidase A removes amino acid residues from the COOH terminus. A control experiment in which buffer that contained no carboxypeptidase

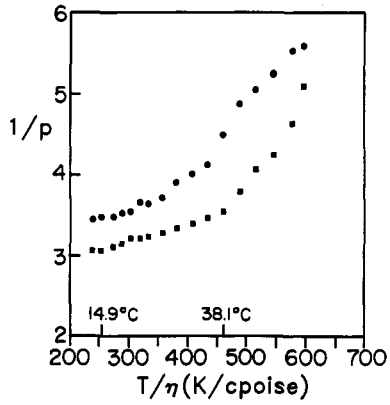

FIGURE 3: Perrin plots of 2 μM samples of AD-TM (■) and AD-NPTM (●) in 150 mM KCl/20 mM Mops, pH 7.0. Polarization values of AD were measured at various temperatures. Excitation was at 360 nm. Emission was detected at 510 nm.

A was added to Py-TM confirmed that photobleaching was not a factor in the results. The "before" and "after" spectra were identical with each other for the control. The absence of photobleaching is attributable to the low intensity of the excitation source (a pulsed 8.3-W Xe discharge lamp) in the system.

AD-TM and AD-NPTM. Figure 3 shows Perrin plots for AD-TM and AD-NPTM in solutions near physiological ionic strength. The AD-TM data agree well with those of Lehrer and Ishii (1988), with extrapolation to $T/\eta = 0$ giving limiting polarization values of 0.40 in both cases. The lower polarization values (higher $1/p$ values in Figure 3) for AD attached to Cys-190 of NPTM relative to intact TM at all temperatures studied show AD to be less constrained on AD-NPTM than on AD-TM. This is consistent with the Py results in suggesting that the COOH terminus in some way restrains the Cys-190 region. Removal of the COOH terminus with carboxypeptidase A relaxes these restraints. Similar Perrin plots were obtained for AD-labeled samples in low ionic strength solutions (results not shown).

Unlike the Py-TM case in moderate concentration salt solution, AD-TM polarization behavior with temperature does not show a dramatic anomaly near 38 °C. This probably reflects differences in the barriers to excimer formation for pyrenes (one excited) on adjacent TM chains, relative to those for independent rotation of AD levels at their respective Cys-190 sites of attachment. Further comment would require additional relevant data.

As with Py-TM, the effects of removal of the COOH terminus of AD-TM at 25 °C in a solution at physiological ionic strength can be followed directly by measurement of polarization at different time intervals (Figure 4, points on the graph show the average change in polarization ± one standard deviation for three measurements on each sample). The small but significant drop in polarization again indicates a greater degree of flexibility of the probe at Cys-190 in the absence of the COOH terminus.

Tyrosine CD of TM and NPTM. CD from tyrosine residues along the TM chain should provide information that is more general than fluorescence from a probe at a single location on the chain. At the same time, it will be more specific than polypeptide backbone CD measured near 222 nm. Of the six tyrosines in each TM chain, five are found in the region from Tyr-162 to the COOH terminus, and four are found at positions beyond Cys-190.

Samples of oxidized TM and NPTM in physiological ionic strength solutions were placed in 5-mm cells, and their ellipticities at 280 nm were followed as a function of temperature

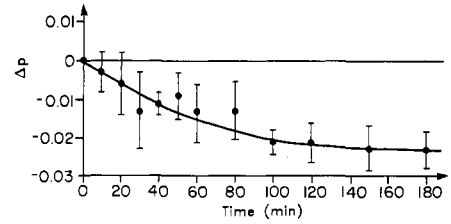

FIGURE 4: Decrease of AD-TM fluorescence polarization on digestion with carboxypeptidase A. Polarization from 2 μM AD-TM was measured at various times after the addition of carboxypeptidase A to the sample in a 1/50 (w/w) ratio to TM and subtracted from polarization values recorded for a similar sample to which only buffer had been added. Conditions were as in Figure 3. Temperature = 25 °C. Data are presented as mean ± standard deviation of three polarization measurements at a single temperature.

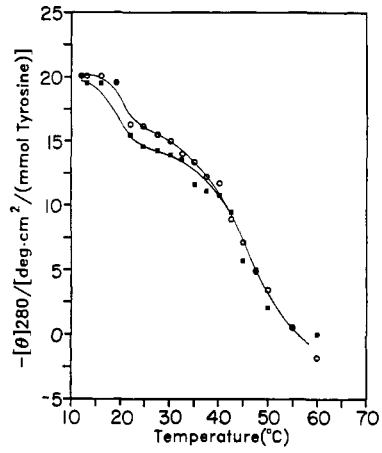

FIGURE 5: Tyrosine CD melting curves. Data were recorded at 280 nm for oxidized samples of TM (○) and NPTM (■), at 1.8 and 1.2 mg/mL, respectively, in 150 mM KCl/10 mM Tris-HCl, pH 8.0.

(Figure 5). The profiles closely resemble those reported in thermal denaturation studies of oxidized TM monitored by peptide backbone CD at 222 nm (Lehrer, 1978) and by tyrosine fluorescence polarization (Satoh & Mihashi, 1972; Lehrer, 1978). There is a pretransition that is essentially complete by 30 °C followed by a main unfolding transition centered near 45 °C. Up to the end of the pretransition, the signal from NPTM falls further and at a few degrees lower temperature than does the signal from TM. The thermal denaturation curves for TM and NPTM converge in the region of the main unfolding transition.

The lower tyrosine ellipticities that we observe in the room temperature region are consistent with the lower α-helical content, estimated from ellipticities at 222 nm, of NPTM relative to TM at those temperatures (Mak & Smillie, 1981a). At temperatures near to and above 45 °C, the thermal denaturation curves determined for TM and NPTM using ellipticities at 222 nm are indistinguishable (Mak & Smillie, 1981a), as is the case for our tyrosine CD profiles. Our conclusions on the relative stability of NPTM and intact tropomyosin from tyrosine CD studies are essentially the same as those based on peptide bond CD data. While the overall stability of NPTM is nearly identical with that of intact tropomyosin, some destabilization is indicated by the somewhat lower ellipticities near room temperature. In their thermal denaturation profiles for reduced tropomyosin, Mak and Smillie (1981a) also report about a 2 °C lower temperature at which NPTM is 50% unfolded relative to intact tropomyosin. Because near-ultraviolet CD signals from proteins are much weaker than those at 222 nm, the inherent noise in our tyrosine CD data may not allow significance to be given to such a small change.

The CD signal at 280 nm is an average of the responses of the six tyrosine residues on each TM chain (Bullard et al., 1976; Nagy, 1977; Holtzer et al., 1989). The tyrosines are found at positions 60, 162, 214, 221, 261, and 267 (Lewis & Smillie, 1980). As five of the tyrosine residues are found in the COOH-terminal half of the molecule, it is likely that the more pronounced pretransition of NPTM (Figure 5) is a result of a decreased rigidity of the coiled-coil structure in that half of the protein relative to what is found in intact TM.

These results, like those from AD-labeled TM and NPTM, are consistent with a model in which the COOH-terminal half of the coiled-coil structure for NPTM is less constrained than for intact TM. The absence in the tyrosyl CD data of an effect that correlates directly with the anomalous peak in excimer emission intensity for Py-TM near 38 °C (Figure 1a) is the result of a combination of the distance of the nearest reporting tyrosine from Cys-190 and the extent of the region actually involved in the pretransition. If the most significant structural change occurs near Cys-190, then a probe at that particular residue (e.g., Py) could provide a more definitive signal than probes (e.g., the tyrosyl rings in the case of Figure 5) 24 or more residues away.

Conclusion

The main conclusion from this study is that the COOH terminus of TM is required to hold the COOH-terminal half of the molecule, the Cys-190 region in particular, in a specific conformation. Removal of a few amino acid residues from the COOH-terminal is readily detectable at Cys-190, the site of attachment of pyrene and acrylodan labels used in this study, some 90 amino acyl positions away. This long-range communication between distant regions on TM could well influence how TM participates in regulation of striated muscle contraction. Our results show that this communication can occur independently of the binding of TM to F-actin or to troponin T, which is thought to interact with TM at sites near to Cys-190 and at the COOH-terminal of TM (Mak & Smillie, 1981b).

Registry No. Cys, 52-90-4.

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