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Interaction of iodoacetamidofluorescein-labelled tropomyosin with deoxyribonuclease I

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5-Iodoacetamidofluorescein (IAF) reacted with rabbit cardiac muscle tropomyosin (TM) to yield a highly fluorescent product, IAF-TM. The extent of labelling reached one fluorescein group per TM molecule in solutions at pH 8.5. While fluorescence polarization values for IAF-TM solutions were unaffected by the presence or absence of KCl, addition of pancreatic deoxyribonuclease I (DNase I) resulted in a 10% drop, suggestive of a greater freedom of motion of the fluorescein label in the presence of DNase I. Furthermore, a 15% increase in slopes of Stern-Volmer plots for IAF-TM in the presence of DNase I demonstrated a greater susceptibility of the fluorescein group to dynamic quenching by iodide. These results suggest that interaction between DNase I and TM produces a localized unfolding of the coiled coil near the IAF reactive site on TM.

Tropomyosin; Deoxyribonuclease I; Fluorescence polarization; Fluorescence quenching

1. INTRODUCTION

TM is a highly helical actin-binding protein found in muscle and non-muscle tissues (reviews [1,2]). Rabbit cardiac muscle TM consists of two identical polypeptide chains [3], each of 33 kDa, coiled around each other to form a rod some 41 nm long. It binds along the length of F-actin filaments and functions with troponin to confer calcium sensitivity to the interaction of myosin with actin (review [4]). Rabbit cardiac TM has a single sulfhydryl group at Cys-190 of each polypeptide chain. This residue and its homologs on other types of TM have often been used as targets for attachment of spectroscopic reporter groups [5–8]. These different labels may introduce some structural alterations into the coiled coil at the site of labelling [9], but each has provided some information about the structure of TM or about its interactions with other proteins.

Here, we report our findings on the fluorescence properties of TM labelled with IAF. The effects of labelling may be assessed in terms of the understood properties of unlabelled TM. New information is made available on the nature of the interaction of cardiac TM with DNase I, which has been reported to bind to muscle TM and inhibit its ability to polymerize in an end-to-end fashion in low ionic strength solutions [10].

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Abbreviations: IAF, 5-iodoacetamidofluorescein; TM, tropomyosin; DNase I, deoxyribonuclease I; DTT, DL-dithiothreitol; Mops, 3-(*N*-morpholino)propanesulfonic acid

2. MATERIALS AND METHODS

Rabbit cardiac TM was prepared according to Smillie [11]. Our preparations were judged to be homogeneous by SDS-polyacrylamide gel electrophoresis [12]. Ovalbumin (Sigma) and the bovine pancreatic enzymes DNase I (Calbiochem), trypsin (TPCK-treated, Sigma) and chymotrypsin (Worthington) were purchased and used without further treatment.

Concentrations of unlabelled proteins were determined spectrophotometrically using absorption coefficients (in $ml \cdot mg^{-1} \cdot cm^{-1}$) at 280 nm of 0.75 for ovalbumin [13], 1.23 for

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DNase I [14], 1.43 for trypsin [15], 2.04 for chymotrypsin [16], and at 277 nm of 0.345 for cardiac TM [17]. Concentrations of IAF-TM solutions were determined using the procedure of Bradford [18] modified for use with Bio-Rad protein staining reagent. Standard curves were prepared using unlabelled samples of cardiac TM.

Absorbance measurements were made with a Perkin-Elmer Lambda 4B spectrophotometer. Steady-state fluorescence data were collected using a Perkin-Elmer LS 5B luminescence spectrometer connected to a Perkin-Elmer 7500 professional computer. A circulating water bath (Haake) was used to maintain samples at 20°C.

Fluorescence quenching data were analysed using the Stern-Volmer equation [19]:

$$F_0/F = 1 + K_{sv}[I^-]$$

where F_0 and F are sample fluorescence intensities in the absence and presence, respectively, of iodide, the quencher used in these studies, and K_{sv} is the Stern-Volmer constant, measured as the slope of a plot of F_0/F vs iodide concentration, $[I^-]$. K_{sv} is the product of the fluorescence lifetime of the chromophore in the absence of quencher and the second-order rate constant that characterizes the dynamic quenching process. Where quoted, K_{sv} values and their standard deviations were determined by linear regression analysis.

Fluorescence polarization values, $p = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, were determined 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 account of the grating correction factor for the instrument.

Prior to reaction with IAF, TM (5 mg/ml) was incubated under reducing conditions, 150 mM KCl, 20 mM dithiothreitol (DTT), 20 mM Mops buffer (pH 7.0), for 4 h at 37°C, subjected to isoelectric precipitation at pH 4.6, pelleted at 15000 × g, and resuspended in 150 mM KCl, 20 mM Mops (pH 7.0), in the presence or absence of 6 M guanidine-HCl. The pH of the solution was raised to 8.5 by dropwise addition of 0.1 M NaOH and a 10–30-fold molar excess of IAF (dissolved in a minimal volume of *N,N*-dimethylformamide) was added. The reaction was allowed to proceed in the dark at room temperature for 1 h, with occasional readjustment of the pH to 8.5. An additional aliquot of IAF was added and the reaction allowed to proceed for another hour. The pH of the solution was then restored to 7.0 and it was put on to dialyse overnight at 4°C vs 150 mM KCl, 20 mM Mops (pH 7.0). Final traces of excess IAF were removed by passage of the sample through a Bio-Gel P2 desalting column.

The degree of labelling of TM was quantitated by independent determination of the amount of label (ϵ = 75000 $M^{-1} \cdot cm^{-1}$ at 495 nm [20]) and the amount of protein [18] present in each IAF-TM preparation.

Tryptic and chymotryptic digestion of TM and separation of the peptides by thin-layer electrophoresis followed by thin-layer chromatography were performed as in [21]. Fluorescent spots were viewed under a hand-held ultraviolet lamp.

Viscosity measurements were performed in a thermostatted water bath held at 27°C in a Cannon-Manning semi-micro viscosimeter (size 75), having a flow time for protein-free buffer of about 2 min.

3. RESULTS AND DISCUSSION

The extent of incorporation of the fluorescein label into TM varied with reaction conditions, particularly pH. At pH 7 at room temperature in nondenaturing buffer, a typical extent of reaction was 0.2 fluorescein groups incorporated per TM molecule. This could be elevated to about 0.5 fluoresceins per TM if the reaction was performed in 6 M guanidine-HCl at 37°C. If the pH of the reaction mixture was maintained at 8.5, whether or not denaturant was present, about one fluorescein group could be attached to each TM molecule at room temperature. On excitation at 490 nm, all of the IAF-TMs produced would emit green light with a broad and featureless emission maximum near 517 nm.

The labelled protein migrated as a single fluorescent band when subjected to polyacrylamide gel electrophoresis in the presence of SDS. It co-migrated with unlabelled cardiac TM. Both tryptic and chymotryptic digestion of IAF-TM having one fluorescein per TM produced several ninhydrin-sensitive spots on cellulose sheets when subjected to thin-layer electrophoresis and subsequently to thin-layer chromatography in an orthogonal direction. However, only one fluorescent spot was observed from each of these digests. We assume, given the selectivity of IAF for sulfhydryl groups, the site of labelling to be Cys-190.

The viscosity of IAF-TM (0.9 mg/ml) relative to 10 mM Mops (pH 7.0) (the solvent used) was 1.11. Addition of salt is expected to reduce this value [17], and we did observe that addition of KCl up to 300 mM could reduce this relative viscosity to 1.00. However, the relative viscosity of an unlabelled TM sample in 10 mM Mops (pH 7.0) was found to be 2.04. Clearly, the label does affect the ability of TM to interact with itself in an end-to-end manner. Similar effects on TM behaviour have been observed as a result of other modifications of Cys-190 [9,22].

As expected from the viscosity behaviour of IAF-TM, fluorescence polarization values were unaffected by the presence or absence of KCl up to 300 mM. In contrast, addition of DNase I brought about a significant drop in polarization of IAF-TM fluorescence in both low and high ionic strength solutions (up to 0.6 M KCl) (fig.1). The effect of DNase I levels off as it nears a 2-fold

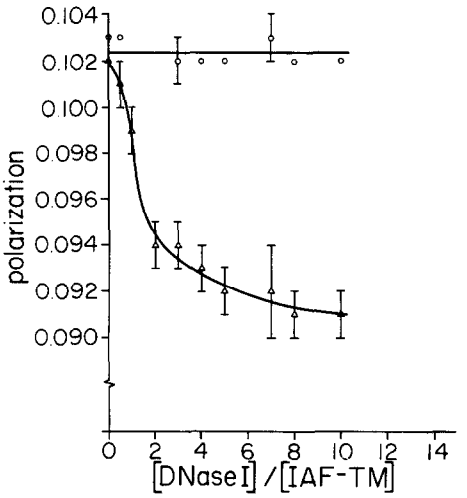

Fig.1. Reduction in polarization of IAF-TM fluorescence on addition of DNase I. To each of two solutions of 0.1 μM IAF-TM in 150 mM KCl, 20 mM Mops (pH 7.0) were added equal volumes of a concentrated solution of DNase I (△) or protein-free buffer (○). Each point is the average of 6 determinations. Error bars indicate standard deviations (only 2 representative error bars are shown for the top line). The points on the top line demonstrate that dilution effects were minimal. Excitation was at 490 nm and emission at 515 nm, with 5 nm bandpass slits at both monochromators.

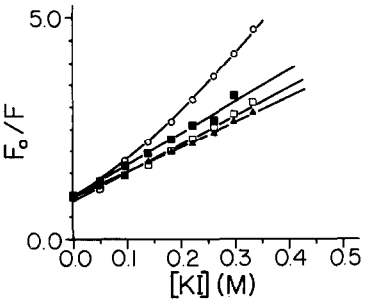

Fig.2. Stern-Volmer plots for iodide quenching of IAF-TM. Two identical solutions were required for each quenching study, one to which was added aliquots of freshly prepared 2 M KI and the other to which was added equal volumes of 2 M KCl. These solutions, respectively, gave fluorescence values F and F_0 for each addition. Excitation was at 490 nm and emission at 515 nm, with 5 nm bandpass slits at both monochromators. (▲) 0.1 μM IAF-TM in 150 mM KCl, 20 mM Mops, pH 7.0. (□) 0.4 μM DNase I and 0.1 μM IAF-TM in 150 mM KCl, 20 mM Mops, pH 7.0. (■) 0.1 μM IAF-TM in 6 M guanidine-HCl, 150 mM KCl, 20 mM Mops, pH 7.0. (○) 0.1 μM IAF in 150 mM KCl, 20 mM Mops, pH 7.0.

molar excess over TM, in agreement with the stoichiometry of DNase I:TM observed in a precipitable complex formed between the two proteins [10]. We recently observed a similar reduction in polarization values of muscle TMs modified at Cys-190 with a coumarin-based sulfhydryl probe [22]. The drop in polarization indicates a greater freedom of motion of the fluorescent label, perhaps as a result of localized unfolding of the TM coiled coil.

In support of this hypothesis, fluorescence from the fluorescein label on TM was found to become more susceptible to quenching by added I^- in the presence of DNase I (fig.2). There is a steady increase in the slopes of the Stern-Volmer plots in the order: IAF-TM in neutral buffer (K_{sv} = 5.67 ± 0.10), IAF-TM in the presence of DNase I in neutral buffer (K_{sv} = 6.53 ± 0.22), IAF-TM in 6 M guanidine-HCl (K_{sv} = 7.21 ± 0.32), IAF alone in neutral buffer (K_{sv} = 11.3 ± 0.5, upward curvature of this particular plot suggests that a more complex analysis in terms of both dynamic and static quenching effects is warranted).

To verify that the effects of DNase I on polarization and quenching of fluorescence of IAF-TM are not general for the addition of any protein at random, ovalbumin was used in place of DNase I. Ovalbumin has no known attraction for TM and did not affect either fluorescence polarization or fluorescence quenching (K_{sv} = 5.42 ± 0.18) of IAF-TM when present at a 4-fold molar excess over TM.

DNase I is known to interact with G-actin to form a 1:1 complex that can neither hydrolyse DNA nor polymerize [23]. It also has been reported to disrupt the end-to-end polymerization of TM in low ionic strength solutions and to form a precipitable complex in which DNase I is present in a 2-fold molar excess over TM [10]. Although a generally accepted physiological reason for the effects of DNase I on these two contractile proteins has not been proposed, the consequences of the interactions provide an insight into the structure and self-recognition properties of actin and TM. Here, we have provided evidence that the interaction of DNase I with TM results in local unfolding of the TM coiled coil. As a not uncommon result of modification of Cys-190 on TM is reduced end-to-end polymerizability (present data and [9,22]), we suggest that uncoiling of TM near Cys-190 may explain the effects of DNase I on the polymerizability of TM.

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