Crosslinking of tropomyosin to myosin subfragment-1 in reconstituted rabbit skeletal thin filaments

Terence Tao and Mark Lamkin

Department of Muscle Research, Boston Biomedical Research Institute, 20 Staniford Street, Boston MA 02114 and *Department of Neurology, Harvard Medical School, Boston, MA, USA*

Received 20 January 1984

Rabbit skeletal tropomyosin was labeled with the bifunctional photoactivatable crosslinker *N*-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate. After irradiating the rigor complex composed of myosin subfragment-1, crosslinker-labeled tropomyosin, and F-actin, a crosslinked product was formed. This product was identified as a 1:1 adduct of tropomyosin and subfragment-1. This finding is in support of recent structural studies which suggest that tropomyosin and subfragment-1 are in close proximity to each other, and may be relevant to the mechanism of thin filament regulation.

Skeletal muscle *Tropomyosin* *Myosin* *Photocrosslinking*

1. INTRODUCTION

Although it is well established that the contraction of skeletal muscle is regulated by Ca^{2+}, the mechanism of this regulation process is still unclear. Early structural work suggested that regulation is achieved by Tm sterically blocking or unblocking the interaction between the myosin heads and the actin subunits, depending on whether or not Ca^{2+} were bound to Tm [1–3]. The validity of this so-called 'steric blocking model' was questioned when it was reported that Tm and S1 are located on opposite sides of the actin helix [4], and therefore cannot interact with each other sterically. However, more recent electron microscopic image reconstruction studies suggest that interaction between Tm and S1 can occur in the S1·Tm·F-actin complex [5–8].

Regardless of the actual geometry of the S1·Tm·F-actin complex, it is clear that the proximity of Tm and S1 bears direct relevance to the mechanism of thin filament regulation. In view of the fact that only structural studies had addressed this important question, it seems desirable to carry out further studies on this system in solution. Using the technique of photoactivated crosslinking, we found that Tm can be crosslinked to S1 in the S1·Tm·F-actin rigor complex. The length of the crosslinker is such that the distance between the attachment points in Tm and in S1 is not likely to be more than 12 Å.

Abbreviations· Tm, rabbit skeletal tropomyosin; S1, chymotryptic myosin subfragment-1; Suc-ANPH, *N*-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate; ANPH-Tm, Suc-ANPH-labeled Tm

2. MATERIALS AND METHODS

All muscle proteins were prepared from rabbit skeletal muscle; Tm was prepared as in [9], actin according to [10], myosin as in [11], and chymotryptic S1 according to [12].

Lysine residues of Tm were labeled with Suc-ANPH (Pierce, Milwaukee, WI) by incubating the protein (in 20 mM phosphate, 1 mM EDTA, 0.12 M NaCl, pH 7.0) with 1.2 mol Suc-ANPH (added from a stock solution in dimethylformamide) per mol Tm at 37°C for 3 h. The reaction was quenched with excess lysine, and the excess reagents removed by dialysis. Using the adduct between Suc-ANPH and *N*-acetyllysine as a model

Published by Elsevier Science Publishers B.V
00145793/84/$3.00

compound, the extinction coefficient of the ANPH group was estimated to be 4800 $M^{-1} \cdot cm^{-1}$ at 480 nm. From this extinction coefficient, it was determined that an average of 1.2 mol of the ANPH moiety was covalently attached to 1 mol of dimeric Tm (M_r = 66000).

ANPH-Tm was ^{14}C-labeled by the method of reductive methylation, which methylates lysine residues of proteins without changing the charge of the ϵ-amino group [13]. ANPH-Tm (2 mg/ml, in 20 mM Hepes, 0.1 M NaCl) was treated with 5 mM $NaCNBH_3$ (Aldrich), followed by 1 mM [^{14}C]formaldehyde (55 mCi/mmol, New England Nuclear). The reaction was allowed to proceed at 4°C for 1 h, before quenching with excess lysine, and then dialyzed to remove excess reagents. We estimated that 0.2 lysine residues were methylated per mol dimeric ANPH-Tm. Similarly, S1 and F-actin were tritium-labeled using [^3H]formaldehyde (100 mCi/mol, New England Nuclear).

Rigor complexes were formed by first adding Tm to F-actin in a medium containing 0.2 mM ATP, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM NaCl, 2 mM Hepes (pH 7.5). S1 was then added, and the mixture allowed to incubate until all the ATP was hydrolyzed. The molar ratio of S1:Tm:actin was 7:1:7. Using the preparative ultracentrifuge, we found that ANPH-[^{14}C]Tm and [^3H]S1 co-sedimented with F-actin to the same extent as unmodified Tm and S1.

3. RESULTS AND DISCUSSION

After irradiating the rigor complex of ANPH-[^{14}C]Tm, [^3H]S1 and unmodified F-actin for 5 min a band with lower electrophoretic mobility than that of S1 heavy chain appeared (fig.1, track 2). A parallel experiment was carried out on an identical sample to which 10 mM $Na_4P_2O_5$ was added in order to dissociate S1 from F-actin. No low-mobility band appeared after irradiation under identical conditions (fig.1, track 3). The gel designated as track 2 in fig.1 was cut into 2-mm slices, and the ^{14}C and ^3H radioactivities associated with each slice were determined. As shown in fig.2a, both ^{14}C and ^3H radioactivities were associated with the low-mobility band. These findings strongly suggest that the low-mobility band corresponds to a photo-induced crosslinking product between ANPH-[^{14}C]Tm and some part of [^3H]S1.

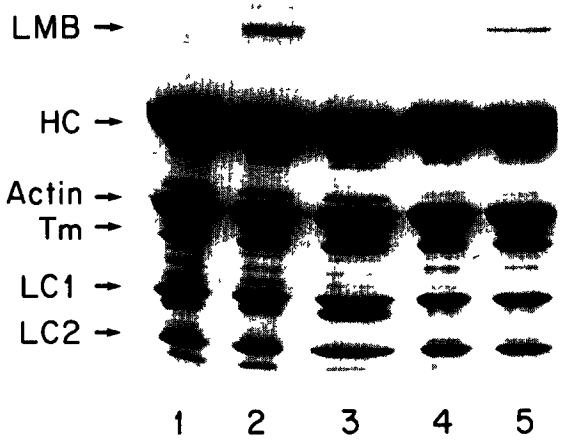

Fig.1. Gradient (6–18%) SDS-polyacrylamide gel electrophoresis of S1·Tm·F-actin rigor complexes; track 1: [^3H]S1·ANPH-[^{14}C]Tm·F-actin, no $Na_2P_2O_4$, before irradiation. Track 2: same as track 1, after 5 min illumination. Track 3: same as track 2, in 10 mM $Na_2P_2O_4$. Track 4: S1·ANPH-[^{14}C]Tm·[^3H]F-actin, no $Na_2P_2O_4$, before irradiation. Track 5: same as track 4, after 5 min irradiation. LC1 and LC2: S1 light chains 1 and 2, respectively. HC: S1 heavy chain; LMB: low-mobility band. Irradiation was carried out at 4°C in a Rayonet RPR-100 'Photochemical Reactor' equipped with 16 '3500' lamps (Southern New England Ultraviolet, Hamden, CT).

In order to determine whether actin is also present in the low-mobility band, the rigor complex of unmodified S1, ANPH-[^{14}C]Tm and [^3H]F-actin was irradiated for 5 min. As before, a low-mobility band appeared when irradiation was carried out in a pyrophosphate-free medium (fig.1, track 5). When the gel corresponding to track 5 in fig.1 was sliced and counted, only ^{14}C radioactivity was found to be associated with the low-mobility band (fig.2b), indicating that actin subunits are not present in the crosslinked product.

The S1:Tm stoichiometry of the crosslinked product was determined as follows: assuming for the time being that the heavy chain of S1 was crosslinked to ANPH-Tm, we have

$$\mu = m_{HC}/m_{Tm}$$

$$= \frac{c_{HC}\,(M_{HC}/C_{HC})}{c_{Tm}\,(M_{Tm}/C_{Tm})}$$

$$= \frac{(c_{HC}/c_{Tm})\,(M_{HC}/M_{Tm})}{(C_{HC}/C_{Tm})}$$

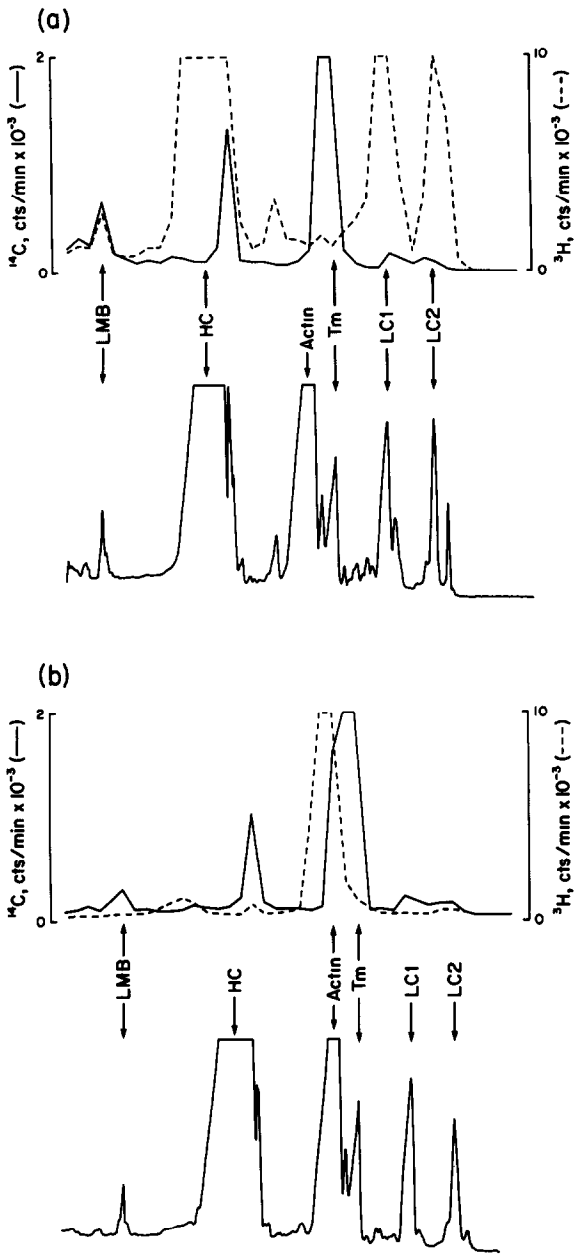

Fig.2. (a) Lower panel: densitometric scan of the Coomassie blue-stained gel designated as track 2 in fig.1 ([^{3}H]S1·ANPH-[^{14}C]Tm·F-actin, irradiated for 5 min in the absence of $Na_2P_2O_4$). Upper panel: distribution of ^{14}C and ^{3}H radioactivities associated with 2-mm slices cut out of the gel designated as track 2 in fig.1. Each gel slice was dried overnight, then solubilized in 125 μl of 30% H_2O_2 for 2 h at 60°C. Protosol (0.5 ml) (New England Nuclear) was then added, followed by 5 ml Betafluor (National Diagnostics, Somerville, NJ). The samples were counted in a Beckman LS-7500 scintillation counter. Counts in the tritium channel were corrected for ^{14}C spillover using ANPH-[^{14}C]Tm as a ^{14}C standard. (b) Lower panel: densitometric scan of the Coomassie blue-stained gel designated as track 5 in fig.1 (S1·ANPH-[^{14}C]Tm·[^{3}H]F-actin, irradiated for 5 min in the absence of $Na_2P_2O_4$). Upper panel: the distribution of ^{14}C and ^{3}H radioactivities associated with 2-mm slices cut out of the gel designated as track 2 in fig.1.

where μ is the molar ratio of S1 heavy chain to Tm monomer in the low-mobility band; m_{HC} and m_{Tm} are moles of heavy chain and Tm monomer, respectively, in the low-mobility band; M_{HC} and M_{Tm} are total moles of heavy chain and of Tm, respectively, in the gel; c_{HC} and c_{Tm} are ^{3}H and ^{14}C counts, respectively, in the low-mobility band; C_{HC} is total ^{3}H counts associated with heavy chain, and C_{Tm} is total ^{14}C counts associated with Tm.

The ratio (M_{HC}/M_{Tm}) is given by the molar ratio at which the proteins were mixed, viz. 3.5, corresponding to 7 mol S1 added to 1 mol Tm dimer. The ratios (c_{HC}/c_{Tm}) and (C_{HC}/C_{Tm}) were readily determined from the data in table 1:

$$c_{HC}/c_{Tm} = 2801/661 = 4.24,$$

$$C_{HC}/C_{Tm} = (234303 + 2801)/(15816 + 1348 + 661) = 13.30$$

Thus,

$$\mu = 1.12.$$

Alternatively, the radioactivities of known amounts of [^{3}H]S1 and ANPH-[^{14}C]Tm were determined (table 1), from which the absolute amounts of heavy chain and Tm in the low-mobility band were obtained:

$$m_{HC} = c_{HC}/(C^{ST}_{HC}/M^{ST}_{HC})$$
$$= c_{HC}/0.76\ (C^{S1}_{ST}/M^{ST}_{S1})$$
$$= 2801\text{ cpm}/0.76\ (182147\text{ cpm}/830\text{ pmol})$$
$$= 16.8\text{ pmol},$$

$$m_{Tm} = c_{Tm}/(C^{ST}_{Tm}/M^{ST}_{Tm})$$
$$= 661\text{ cpm}/(36820\text{ cpm}/852\text{ pmol})$$
$$= 15.4\text{ pmol},$$

where M^{ST}_{HC} is moles of heavy chain in the [^{3}H]S1

Table 1

Radioactivities associated with the bands in the gel for irradiated [^{3}H]S1 · ANPH-[^{14}C]Tm · F-actin

	cpm
^{3}H radioactivity, S1 heavy chain band:	234303
^{3}H radioactivity, S1 light chain bands:	73204
^{3}H radioactivity, low-mobility band:	2801
^{14}C radioactivity, Tm monomer band:	15816
^{14}C radioactivity, unidentified crosslinking band:	1348
^{14}C radioactivity, low-mobility band:	661
^{3}H radioactivity, [^{3}H]S1 standard (830 pmol):	182147
^{14}C radioactivity, ANPH-[^{14}C]Tm standard (852 pmol):	36820

The bands correspond to those shown in fig.2a. Radioactivities were determined according to the method described in the legend to fig.2a. For the standard samples, a known amount of the protein was mixed with a 2 mm piece of protein-free gel slice. Exactly the same procedure described in the legend of fig.2a was then used to measure the radioactivity

standard sample; M_{S1}^{ST} is moles of [^{3}H]S1 standard; C_{HC}^{ST} is ^{3}H counts associated with the heavy chain of the [^{3}H]S1 standard; C_{S1}^{ST} is ^{3}H counts for the [^{3}H]S1 standard; M_{Tm}^{ST} is moles of ANPH-[^{14}C]Tm standard; and C_{Tm}^{ST} is ^{14}C counts for the ANPH-[^{14}C]Tm standard; 0.76 is the fraction of heavy chain ^{3}H counts over total S1 ^{3}H counts. Thus, μ = 1.09, corresponding again to a 1:1 stoichiometry of S1 heavy chain : ANPH-Tm monomer for the crosslinked product.

If it was assumed that the light chains of S1 were crosslinked to ANPH-Tm, then similar calculations yielded a stoichiometry of 6.9 mol light chains per mol ANPH-Tm monomer. This seems unlikely since only 0.6 crosslinkers (the ANPH moiety) were attached per mol monomeric ANPH-Tm. The fraction of monomeric ANPH-Tm molecules with more than 6 ANPH moieties attached would be so small that the probability for one ANPH-Tm monomer to form more than 6 crosslinks simultaneously would be extremely low. Thus, it appears that the crosslinked product is composed of ANPH-Tm monomer and S1 heavy chain at a molar ratio of 1:1.

Our results show that ANPH-Tm can be crosslinked to S1 in the S1 · Tm · F-actin rigor complex. Using a molecular model, we estimated that the chain between the attachment points of the crosslinker extends ~15 Å when fully stretched (note that this is shorter than the reagent itself because the *N*-hydroxylsuccinimidyl group leaves after reaction). However, the probability for the chain to actually adopt this configuration is vanishingly low. If we approximate this chain as a polymethylene chain of the same length (13 carbons), then a statistical analysis (discussed in [14]) yielded a most probable length of 6.2 Å, and a 6% probability for the length to be greater than 12 Å. Thus, we believe that it is not likely for Tm to be more than 12 Å away from S1 in the S1 · Tm · F-actin complex. Our results are therefore compatible with the findings that Tm is in close proximity to S1 in the S1 · Tm · F-actin complex [5–8]. Our results cannot prove, however, that Tm actually makes contact with S1. Further studies using shorter crosslinking reagents are in progress in order to clarify this point.

ACKNOWLEDGEMENTS

We thank Drs John Gergely and Sherwin S. Lehrer for discussion and reviewing the manuscript. This work was supported by the National Institutes of Health (AM21673).

REFERENCES

[1] Huxley, H.E. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 361–376.
[2] Haselgrove, J. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 341–352.
[3] Parry, D.A.D. and Squire, J.M. (1973) J. Mol. Biol. 75, 33–55.
[4] Seymour, J. and O'Brien, E.J. (1980) Nature 283, 680–682.
[5] Taylor, K.A. and Amos, L.A. (1981) J. Mol. Biol. 147, 297–324.
[6] Amos, L.A., Huxley, H.E., Holmes, K.C., Goody, R.S. and Taylor, K.A (1982) Nature 299, 467–469.
[7] Vibert, P. and Craig, R. (1982) J. Mol. Biol. 157, 299–319.
[8] Wakabayashi, T. and Toyoshima, C. (1981) J. Biochem. (Tokyo) 90, 683–701.
[9] Greaser, M. and Gergely, J. (1971) J. Biol. Chem. 246, 4226–4233.

[10] Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
[11] Balint, M., Sreter, F.A., Wolf, I., Nagy, B. and Gergely, J. (1975) J. Biol. Chem. 250, 6168–6177.
[12] Weeds, A.G. and Pope, B. (1977) J Mol. Biol. 111, 129–157.
[13] Jentoft, N. and Dearborn, D.G. (1979) J. Biol. Chem. 254, 4359–4365.
[14] Tanford, C. (1961) Physical Chemistry of Macromolecules, chapter 3, John Wiley and Sons, New York.