[41] Preparation and Characterization of Tetramethylrhodamine-Labeled Myosin

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Fluorescent analog cytochemistry allows specific proteins to be studied in living cells,[1,2] through the microinjection of fluorescent conjugates. The myosin molecule, with its force-generating properties and its potential to undergo rapid reorganizations in living nonmuscle cells, is a particularly attractive target for the application of this technique.

Critical to the success of fluorescent analog cytochemistry is the preparation of fluorescent conjugates that show proper biochemical and spectroscopic properties. Although the myosin molecule and its proteolytic fragments have been fluorescently labeled for various studies *in vitro*,[3] most probes used are not ideal for living cells due to the relatively short excitation and emission wavelengths or the susceptibility to photobleaching. However, these earlier studies did provide useful general information about the chemistry of fluorescent labeling. For example, each skeletal muscle myosin molecule is shown to contain two highly reactive SH-1 groups and two less reactive SH-2 groups. Both SH-1 and SH-2 are located on the heavy chain in the head region. By contrast, each smooth muscle myosin molecule is known to contain eight reactive sulfhydryl groups, among them four that are highly reactive.[4] Two of those are located on the 17-kDa light chain (one on each 17-kDa light chain) and two on the heavy chains in the head region.

The purpose of this chapter is to describe in detail a method for the fluorescent labeling of the myosin sulfhydryl groups with tetramethylrhodamine, a probe with optimal fluorescence properties for applications in living cells. The method of labeling applies to both skeletal and smooth muscle myosins, although the discussion will focus on the smooth muscle myosin.

Labeling of Myosin with Tetramethylrhodamine Iodoacetamide

The method described below uses tetramethylrhodamine iodoacetamide (TRIA) as the reagent for attaching the tetramethylrhodamine fluor-

[1] Y.-L. Wang, *Methods Cell Biol.* **25,** 1 (1982).
[2] Y.-L. Wang, *Methods Cell Biol.* **29,** 1 (1989).
[3] E. Reisler, this series, Vol. 85, p. 84.
[4] N. Nath, S. Nag, and J. C. Seidel, *Biochemistry* **25,** 6169 (1986).

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ophore to the sulfhydryl groups of the myosin molecule. The procedure is similar to that for other iodoacetamides as summarized by Reisler.[3] However, special steps are taken to overcome the problems of the relatively low solubility of TRIA and the difficulty in removing unreacted TRIA from proteins. All the buffers should be prepared at 4° and all steps are performed at 4°.

Day 1

Buffers Required

Resuspension buffer: 2 *M* KCl, 50 m*M* HEPES, 5 m*M* dithiothreitol (DTT), pH 7.5, 5 ml
Dialysis buffer: 0.5 *M* KCl, 10 m*M* HEPES, pH 7.5, 250 ml

Steps

1. Myosin is prepared from standard, published procedures[4a]. It is stored either as precipitates in 70% ammonium sulfate at 4°, or as precipitates in a low-salt buffer and kept frozen in liquid nitrogen. Precipitates of myosin are pelleted down in a Sorvall SS34 rotor at 18,000 rpm for 10 min.

2. Soak the pellet in a small volume of the resuspension buffer for 1-2 hr and disperse the pellet. The resulting concentration of myosin should be 6-10 mg/ml. The solution should be handled very gently because bubbles and foams will rapidly denature the protein. In addition, excessive aeration oxidizes the sulfhydryl groups that are involved in the labeling reaction. The high concentration of DTT in the resuspension buffer serves to protect the sulfhydryl group and to dissociate any disulfide bridges that may be present.

3. Dialyze the solution against the dialysis buffer overnight. DTT must be removed (gradually) by the dialysis prior to the reaction since it reacts with TRIA.

Day 2

Buffers Required

Reaction buffer: 0.5 *M* KCl, 50 m*M* HEPES, pH 8.0, 250 ml
Assembly buffer: 20 m*M* KCl, 20 m*M* PIPES, pH 7.0, 250 ml

Steps

1. Remove myosin solution from the dialysis tubing and clarify in a Beckman (Palo Alto, CA) 50Ti rotor at 20,000 rpm for 30 min. The solution should be handled gently and reaction started without delay.

[4a] I. Ikebe and D. J. Hartshorne, *J. Biol. Chem.* **260**, 13146 (1985).

2. Determine the concentration of myosin by diluting an aliquot appropriately (e.g., 20×) with the dialysis buffer and reading the absorbance at 280 nm. The approximate concentration of gizzard myosin (in mg/ml) can be calculated as absorbance/0.5.[4b] The total amount of myosin is then calculated based on the concentration and the volume.

3. Resuspend 0.5–1 mg TRIA (Molecular Probes, Eugene, OR; stored desiccated at −20°) in 100 μl dimethyl sulfoxide (DMSO). Break down large aggregates and pipette repeatedly to make sure that the reagent is dissolved as much as possible.

4. While stirring vigorously, add the reagent solution slowly into the reaction buffer to obtain a TRIA concentration of 0.1 mg/ml.

5. Clarify the reagent solution in a Beckman 50Ti rotor at 35,000 rpm for 15 min. Even though the reagent solution may appear transparent without centrifugation, there are a large number of undissolved aggregates.

6. The supernatant is carefully removed and an aliquot diluted appropriately for the measurement of the absorbance. The volume required for the reaction, in milliliters, is calculated as (1.4/absorbance at 555 nm) × mg of myosin. For example, 1 ml is required for reacting with 1 mg myosin if the absorbance of the TRIA solution at 555 nm equals 1.4. This calculation is required in order to adjust for the variability in the extent of solubilization of TRIA. The volume of TRIA solution should be larger than that of myosin. This, in combination with the higher concentration of HEPES in the reaction buffer, should bring the pH close to 8.0.

7. The solutions of TRIA and myosin are mixed gently with a pipette and the mixture incubated for 2 hr on ice.

8. The reaction is stopped by passing the solution through a Bio-Beads SM-2 column (Bio-Rad, Richmond, CA). SM-2 is an adsorption medium for removing small, nonpolar organic molecules. It proves to be very effective for the removal of unreacted TRIA molecules. A column of 1 × 8 cm is adequate for up to 20 mg of myosin.

9. Fluorescent fractions that pass through the column are pooled and dialyzed against the assembly buffer for 4 to 15 hr to induce precipitation of myosin.

Day 3

Buffers Required

Resuspension buffer: 2 *M* KCl, 10 m*M* PIPES, pH 7.0, 5 ml

Steps

1. Labeled myosin molecules that precipitate during dialysis are collected by centrifugation in a Sorvall SS34 rotor at 15,000 rpm for 10 min.

[4b] K. M. Trybus and S. Lowey, *J. Biol. Chem.* **259**, 8564 (1984).

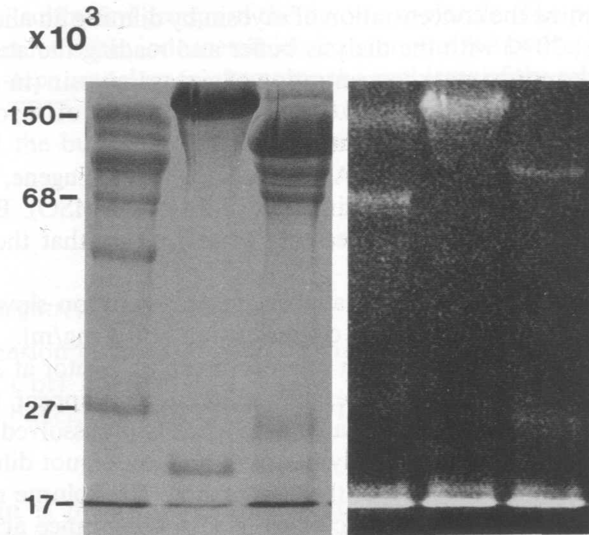

FIG. 1. SDS-polyacrylamide gel electrophoresis of fluorescently labeled myosin (middle lanes), and its proteolytic fragments created by trypsin (left lanes) and papain (right lanes). The left panel shows the pattern of Coomassie Blue staining and the right panel shows the fluorescence. Approximate molecular weights are indicated on the left ($\times 10^3$). (From *J. Cell Biol.* **109,** 1163–1172.)

2. Soak the pellet in a small volume of resuspension buffer for 1–2 hr and resuspend the pellet.
3. Dialyze the solution against an appropriate buffer, clarify before use.

Characterization of TRIA-Labeled Smooth Muscle Myosin

Extent and Sites of Labeling

The labeled myosin should contain no detectable free dye molecules (detection by SDS-PAGE as a fluorescent band near the bromphenol blue tracking dye). The extent of labeling is measured by standard methods: the protein concentration is determined by the Lowry assay, the concentration of tetramethylrhodamine is determined by measuring the absorbance at 555 nm. The molar concentrations of the protein and the fluorophore are then calculated based on the molecular weight of myosin (470,000) and the extinction coefficient of bound tetramethylrhodamine[5] (47,000 M^{-1}cm^{-1}).

[5] R. L. DeBiasio, L.-L. Wang, G. W. Fisher, and D. L. Taylor, *J. Cell Biol.* **107,** 2631 (1988).

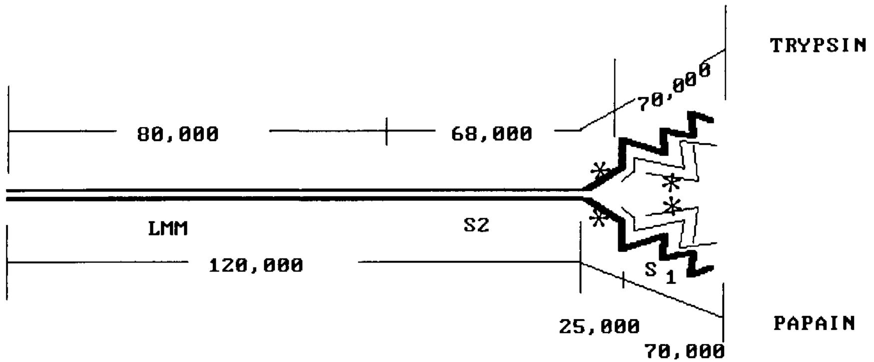

FIG. 2. A diagram of smooth muscle myosin and the sites of limited proteolysis. Primary sites of proteolytic cuts are indicated by long vertical lines, secondary sites are indicated by short vertical lines. The molecular weights of different fragments are indicated. The primary sites of fluorescent labeling by TRIA are indicated by asterisks.

The labeling protocol yields typically a conjugate with four fluorophores per smooth muscle myosin molecule.

The site of labeling can be identified using a combination of PAGE and limited proteolysis. Without proteolysis, the fluorescence is located about 40% on the heavy chain and 60% on the 17-kDa light chain (Fig. 1, middle lanes). The pattern of proteolytic digestion of smooth muscle myosin has been determined previously (Fig. 2). Limited tryptic digestion of the heavy chain creates a 150-kDa fragment that includes the tail portion plus a small region at the C terminus of the head[6] (Fig. 2). Both this fragment and its N-terminal 68-kDa portion (the portion toward the head) are fluorescently labeled (Fig. 1, left lanes). Limited papain digestion of the heavy chain creates a 120-kDa fragment, which represents the myosin tail, and a 95-kDa fragment, which represents the S1 head.[4] Only the latter contains bound fluorophore (Fig. 1, right lanes). Based on these results, one can conclude that the primary sites of labeling are located on the 17-kDa light chain and on the heavy chain near the C terminus of the S1 head (Fig. 2; previously referred to as SH-C). The latter site also corresponds to the highly reactive sulfhydryl group of the skeletal muscle myosin (referred to as SH-1[3,4]).

ATPase Activities and Self-Assembly

ATPase activities of labeled myosin can be assayed with standard methods.[7] Results for unphosphorylated smooth muscle myosin are shown

[6] T. Merianne-Pèpin, D. Mornet, D. Audemard, and R. Kassab, *FEBS Lett.* **159**, 211 (1983).
[7] T. D. Pollard, this series, Vol. 85, p. 123.

TABLE I
EFFECTS OF FLUORESCENT LABELING ON GIZZARD MYOSIN ATPASES[a,b]

Condition	Unlabeled	TRIA labeled	Percentage labeled/unlabeled
K^+-EDTA	983	692	70
Ca^{2+}	564	644	114
Mg^{2+}	6.9	35.4	513
Mg^{2+} [c] (actin activated)	14.4	42.8	297

[a] From *J. Cell Biol.* **109,** 1163–1172.
[b] All activities were measured at 36° and expressed in units of nanomoles per minute per milligram.
[c] Measured in the presence of 0.53 mg/ml F-actin.

in Table I. It is clear that fluorescent labeling has induced a significant change in the ratio of different ATPase activities, including increases in the Mg^{2+}-ATPase (about 5× increase) and the actin-activated Mg^{2+}-ATPase (3× increase). However, the extent of activation of the Mg^{2+}-ATPase by actin filaments is reduced. These observations are consistent with the labeling of the SH-C sulfhydryl group.[4,8]

The self-assembly of labeled myosin is assayed by right-angle light scattering, using a spectrofluorimeter with both the excitation and emission wavelengths set at 340 nm. Myosin is dialyzed into a high-salt buffer (500 m*M* KCl, 0.1 m*M* EDTA, 10 m*M* HEPES, pH 7.5). After clarification, an aliquot is removed and diluted by 15–60 times with an assembly buffer (150 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1 m*M* DTT, 10 m*M* HEPES, pH 7.5) at room temperature to obtain a concentration between 0.1 and 0.5 mg/ml. The intensity of scattered light is monitored until a steady state is reached. Since unassembled myosin molecules also scatter light, a sample is prepared by diluting the same amount of myosin with the high-salt buffer. The intensity of light scattering by unassembled molecules is then subtracted from that by assembled molecules. Results of this assay are shown in Fig. 3. It is clear that both labeled and unlabeled myosins assemble to a similar extent under physiological salt conditions in the absence of ATP (Fig. 3, upper line). When ATP is added to the sample, unlabeled, unphosphorylated myosin shows a dramatic decrease in light scattering (Fig. 3, bottom line), indicating an ATP-induced disassembly.[9,10]

[8] H. Onishi, *J. Biochem. (Tokyo)* **98,** 81 (1985).

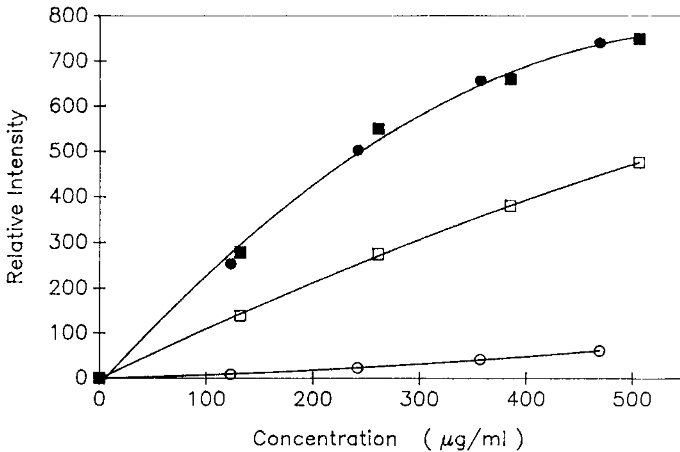

FIG. 3. Assembly and ATP sensitivity of myosin filaments as a function of concentration. Myosin filaments are detected by right-angle light scattering at 340 nm. Fluorescently labeled (squares) or unlabeled (circles) myosin is assembled in an assembly buffer as described in the text, and the steady state intensities measured (filled symbols). ATP is then added to the sample and the steady state intensities measured again (open symbols). Labeled myosin shows normal assembly in the absence of ATP but a reduced sensitivity to ATP. (From *J. Cell Biol.* **109,** 1163–1172.)

Labeled myosin also shows a decrease in light scattering, but the extent is much reduced (Fig. 3, middle line).

Taken together, these results indicate that the fluorescent labeling induces an increase in actin-activated Mg^{2+}-ATPase and a decrease in the extent of ATP-induced disassembly. Thus the conjugate bears some similarities to phosphorylated smooth muscle myosin molecules.

Microinjection of Fluorescently Labeled Myosin

Microinjection of labeled myosin is more difficult compared to the injection of many other proteins, due to the low solubility of myosin under low-salt conditions and the susceptibility of myosin to aggregation. The solution to be microinjected must be thoroughly clarified and handled with great care. We centrifuge the solution with a type 42.2Ti rotor (Beckman) at 25,000 rpm for 20 min. The top 70–80% of the supernatant is carefully removed and used within 48 hr.

[9] H. Suzuki, H. Onishi, K. Takahashi, and S. Watanabe, *J. Biochem. (Tokyo)* **84,** 1529 (1978).

[10] J. Kendrick-Jones, K. A. Taylor, and J. M. Scholey, this series, Vol. 85, p. 364.

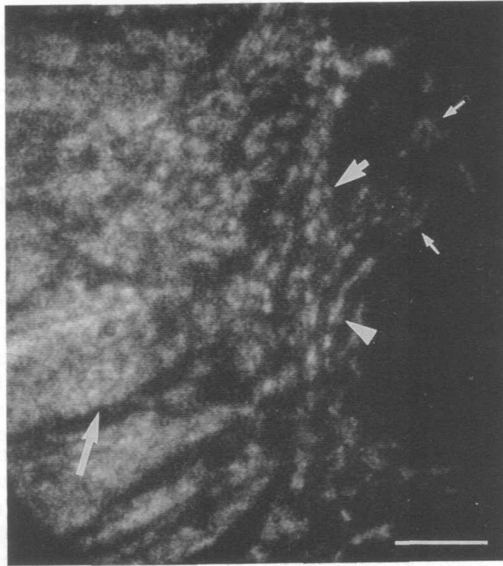

FIG. 4. A living 3T3 cell microinjected with fluorescently labeled myosin, showing clearly resolved beads near the edge of the cell. Many beads appear elongated or filamentous (arrowhead). Near a lamellipodium, myosin beads are sparse, indistinct, and apparently poorly organized (small arrows). Further behind the lamellipodium, the beads are arranged along curved fibers that often seem to cross or merge with each other (medium arrow). The fibers appear tightly packed and laterally associated in a more central area (large arrow). (Bar: 5 μm) (From *J. Cell Biol.* **109,** 1163–1172.)

An injection solution of 450 m*M* KCl, 2 m*M* PIPES, pH 7.0, has been used for microinjection. The high-salt concentration is required for maintaining the solubility of myosin. In addition, a continuous flow is maintained to avoid the plugging of needle tips. As soon as the flow stops, the salt concentration near the tip decreases due to diffusion and myosin molecules assemble rapidly. The microinjection of high-salt buffer does induce transient disruptions in cellular morphology and place a limitation on the amount of myosin that can be delivered. Therefore, in order to obtain a detectable signal with a small volume of microinjection, it is important to keep myosin at a relatively high concentration during microinjection. We have routinely used concentrations between 5 and 9 mg/ml.

A properly injected cell recovers within 2–3 hr of microinjection and should have a phase morphology indistinguishable from that of uninjected cells. Fluorescent myosin should become incorporated into stress fibers in a punctate pattern. In addition, near the cell periphery, where the cyto-

plasm is very thin, myosin molecules are observed as small beadlike structures with an apparent average length of 0.73 μm (Fig. 4). The beadlike structures most likely represent single bipolar filaments.

Conclusion

This chapter describes a relatively simple method for preparing a rhodamine conjugate of myosin. The conjugate maintains many properties of myosin and is readily incorporated into physiological structures. The fluorescence properties of tetramethylrhodamine are ideal for cellular studies, especially those involving time-lapse recordings. However, there are also limitations with the present conjugate. The conjugate behaves somewhat similar to phosphorylated myosin molecules, and may not be useful for studying the effect of phosphorylation or the regulation of assembly. In addition, the labeling of both heavy and light chains may cause ambiguities in some studies.

Several alternative methods may be used to complement the present approach. For example, fluorescently labeled myosin light chains have been prepared and shown to colocalize with myosin-containing structures after microinjection.[11,12] The light chains are much easier to microinject and may be useful in studies where light chains must be examined specifically. Alternatively, it may be possible to label preferentially the heavy chain by blocking the highly reactive sites on light chains with a reversible agent, such as 5,5′-dithiobis(2-nitrobenzoic acid), before the fluorescent labeling (unpublished observations). Following the reaction, the fluorescent conjugate is treated with DTT to recover the sulfhydryl groups on the light chains. Finally, DeBiasio *et al.*[5] have used a slightly different approach for labeling whole myosin, by carrying out the reaction in a physiological salt, ATP-containing buffer which maintains unphosphorylated smooth muscle myosin in a folded conformation.[9,10] It appears that the assembly of such conjugates may have an improved sensitivity to ATP compared to the present preparation. These different approaches have different advantages and disadvantages and can be selected based on the purpose of the study.

[11] B. Mittal, J. M. Sanger, and J. W. Sanger, *J. Cell Biol.* **105**, 1753 (1987).

[12] N. M. McKenna, C. S. Johnson, M. E. Konkel, and Y.-L. Wang, *in* "Cellular and Molecular Biology of Muscle Development" (L. H. Kedes and F. E. Stockdale, eds.), p. 237. Alan R. Liss, New York, 1989.