# Equilibration and Exchange of Fluorescently Labeled Molecules in Skeletal Muscle Fibers Studied Using Confocal Microscopy

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Diffusion of molecules into skinned muscle fibers is often necessary when studying muscle contraction and its regulation. Usually, it is assumed that diffusion of molecules is fast also in the structured system of a muscle fiber, and that equilibration is reached within minutes or at most hours.

One method to study not only equilibration, but also the time dependent distribution and the binding dynamics of fluorescently labeled molecules inside a muscle fiber is confocal laser fluorescence microscopy. Because of the principle of confocal imaging, one can obtain optical sections of a muscle fiber even under physiological conditions and with reasonable time and spatial resolution. In the present study, we used confocal microscopy to follow the equilibration of several fluorescently labeled substances in chemically skinned single muscle fibers of rabbit psoas. The investigation concerned mainly two questions:

- What causes the equilibration time for molecules of the same size and type to be different from minutes to days?
- Is the high affinity binding of some muscle-specific molecules readily reversible?

## Effects of molecular weight on the rate of equilibration of different fluorescently labeled molecules

In a previous study (Kraft et al., 1993), we determined the time required to reach homogeneous distribution throughout muscle fibers for various fluorescently labeled molecules under near physiological conditions. We found no correlation between time for equilibration and the molecular weight of the molecules. Even very large molecules like Rh-pyruvate kinase ($m$ = 237 kDa) reach equilibrium within a few minutes, whereas others of similar size need several days to reach a homogeneous distribution throughout the whole fiber (e.g., Rh-NEM-S1, c.f. Kraft et al., 1993).

To investigate the phenomenon of slow equilibration, we compared the rate of equilibration, the final distribution, and the final concentration (i.e., fluorescence intensity) of the non-muscle-specific antibody TxR DaM-IgG and the actin-specific TRITC-labeled IgG inside muscle fibers.

Although TxR DaM-IgG and TRITC-anti-actin IgG are molecules of the same shape and molecular weight, equilibration of muscle fibers with these molecules differs greatly: (i) The non-muscle-specific TxR DaM reaches homogeneous distribution within 2–3 min, whereas the actin-specific IgG needs at least 2 days for equilibration. (ii) Because of its binding to actin, the final fluorescence intensity inside the fiber is much higher after equilibration of the actin-specific antibody than for the non-muscle-specific DaM-IgG, which shows only little accumulation within the structured system. (iii) The non-muscle-specific DaM-IgG could be removed within a few minutes during incubation of the fiber in relaxing solution, whereas for the actin-specific IgG we found no detectible decrease in fluorescence intensity even after several hours of incubation in relaxing solution, indicating rather high affinity binding of this antibody to its target area. From these observations, it appears that several factors may contribute to the slow equilibration:

- In the structured system of a muscle fiber, we are not dealing with mere diffusion but, instead, with movement of the molecules that also involves binding and dissociation to/from more or less specific sites within the network of the sarcomeric structure (Maughan and Lord, 1988; Gershon et al., 1985).
- Once a substance binds specifically and with high affinity to sites inside the fiber, even if diffusion itself was unchanged, the saturation of all binding sites would take some time because a large number of molecules is required.
- The time for equilibration might also be limited by the accessibility of the binding sites and by obstruction effects, e.g., within the filament lattice (Wegmann et al., 1992). Such obstruction could well be enhanced by binding of the molecules within the myofilament lattice.

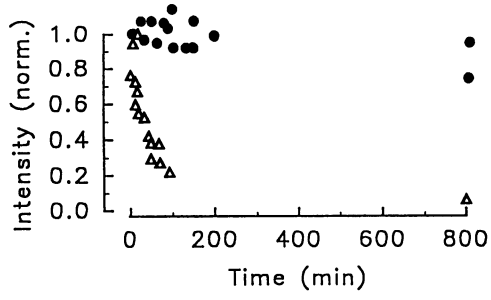


FIGURE 1 Time course of the disappearance of Rh-troponin from the fiber upon incubation in relaxing solution with (△) and without (●) unlabeled troponin. Fluorescence intensity plotted versus time. Fluorescence normalized with respect to maximum intensity at end of preequilibration with Rh-troponin.

## EXCHANGE OF MOLECULES BOUND TO THE THIN FILAMENT

We used confocal microscopy also to study the dynamics of the binding of proteins such as Rh-troponin (Rh-Tn) and Rh-creatine kinase (Rh-M-CK) after equilibration by comparing the time course of spontaneous decrease in fluorescence intensity with the time course of replacement of the proteins by unlabeled molecules in a chase-experiment. After equilibration with the respective fluorescently labeled protein, the fibers were incubated in different ways. (1) Incubation occurred in relaxing solution without any Tn or M-CK present. Any decrease in fluorescence intensity would indicate spontaneous loss of Rh-Tn or Rh-M-CK, respectively, which would be expected if these proteins bind only with low affinity or nonspecifically. (2) Alternatively, fibers were incubated in solution containing excess of unlabelled Tn or M-CK. The decrease in fluorescence intensity in this case reflects mainly the replacement of bound fluorescent molecules by unlabeled Tn or M-CK, respectively. As an example, Fig. 1 shows the results for Rh-Tn. Although Rh-Tn appears to be bound to actin rather tightly, it can be exchanged by unlabeled molecules within a couple of hours, indicating a significant dissociation rate of the molecule. Obviously, the exchange of intrinsic fiber proteins by analogs is a very mild method to introduce modified proteins into muscle fibers, but it still takes quite a long time until the exchange is complete.

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