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Application of Angle-Resolved Fluorescence Depolarization in Muscle Research

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Angle-resolved fluorescence depolarization (AFD) experiments have been used for over a decade in studies of fluorescent molecules in macroscopically aligned systems such as lipid bilayers and stretched polymer films. The importance of this technique lies in the fact that it affords the determination of both the second- and the fourth-rank order parameters of the orientational distribution of the probe molecules in the sample. Here we apply the technique to the study of the orientational distribution of crossbridges in muscle fibers. This orientational distribution is particularly relevant in muscle research, as crossbridge rotation is commonly regarded to be the driving mechanism in force development. An unfortunate consequence of the fact that the crossbridges have an average orientation of approximately 45° relative to the fiber axis is that the values of the second-rank order parameter $\langle P_2 \rangle$ of the crossbridge distribution are close to 0. Therefore, knowledge of $\langle P_4 \rangle$ is essential for a reliable reconstruction of the form of the distribution function. AFD of dye-labeled muscle was measured under rigor and relaxation conditions. The results indicate that no significant changes in depolarization take place upon a transition from the rigor to the relaxed state in the muscle and seem not to support the rotating crossbridge model, which postulates a clear change of orientation of the crossbridges.

KEY WORDS: Angle-resolved fluorescence depolarization; muscle fibers; rotating crossbridge model.

INTRODUCTION

It is generally accepted that in a contracting muscle, actin and myosin filaments slide past each other without changing length. The rotating crossbridge model [1,2] suggests that the sliding of the filaments is brought about by a change of orientation of the crossbridges while attached to the actin. To test this hypothesis much effort has been devoted to the characterization of the orientational behavior of myosin crossbridges in the fiber. Many studies of crossbridge orientation indicate that in the rigor state, in the absence of nucleotide, the crossbridges are strongly bound to the actin filament at an angle of about 45°. In contrast, the orientational behavior of the crossbridges in relaxation and activation (when ATP is present in the system) remains a point of controversy [3–5].

Fluorescence and phosphorescence depolarization techniques have been used extensively to characterize the orientational distribution of the crossbridges at different stages of the contractile cycle [6]. However, the abundance of studies failed to produce a consistent pattern of the crossbridge behavior. In fluorescence and phosphorescence depolarization studies using 1,5-I-AEDANS and E5M attached to the cysteine SH1 on the myosin head, a change in the peak position and the width of the orientational distribution is reported [7–9].

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In contrast, studies using fluorescent analogues of ATP suggest that no rotation of the crossbridges takes place [10].

Several reasons may be identified for this lack of consensus. An unfortunate consequence of the fact that the crossbridges have an average orientation of approximately 45° relative to the fiber axis is that the values of the second-rank order parameter $\langle P_2 \rangle$ of the crossbridge distribution are close to 0. Therefore, knowledge of $\langle P_4 \rangle$ is essential for a reliable reconstruction of the form of the distribution function [11,12]. Commonly the fluorescence depolarization of dye labeled muscle fibers is studied in a fixed 90° scattering geometry. Unfortunately it is not feasible to determine $\langle P_4 \rangle$ unambiguously using this scattering geometry [12,13]. As a result, such an experiment cannot be used to discriminate between different models for the orientation of the crossbridges in the fiber. Indeed, it has been shown that distinctly different models can be used to describe the data obtained using the 90° scattering geometry. For instance, the model introduced by Borejdo *et al.* [14] explicitly assumes that the dye molecules attached to the crossbridges in the muscle fiber all have one and the same polar angle β relative to the fiber axis. On the other hand, in the double-cone model used by Thomas and co-workers [7,9], the dye molecules are assumed to lie within a cone, while the axis of this cone lies within a second cone at an orientation β relative to the fiber axis.

Another reason for the confusion lies in the fact that the comparison of results obtained from depolarization experiments is seriously hampered by their explicit dependence on the particular dyes used in the experiment and the transition dipole moments associated with the wavelengths chosen for excitation and emission. In this context it is important to realize that a fluorescence depolarization experiment monitors only the orientations of the transition dipole moments of the dye. Consequently, it is not straightforward to relate the observed depolarization to the orientational order and dynamics of the crossbridges in the muscle fiber.

Here we report a study which attempts to tackle both problems set out above. To this end we have labeled the crossbridge heads in a muscle fiber with fluorescent dyes and carried out angle-resolved fluorescence depolarization (AFD) measurements. This technique has been used successfully for over a decade in studies of fluorescent molecules in macroscopically aligned systems such as lipid bilayers and stretched polymer films [13,15]. The importance of this technique lies in the fact that it affords the unambiguous determination of both the second- and the fourth-rank order parameters of the orientational distribution of the probe molecules in the sample.

The extraction of the orientational distribution of the crossbridge, independent of the dyes and the transition dipole moments used in the experiment, requires a detailed knowledge of the orientations of the transition dipole moments in the dyes and of the orientation and motion of the dye relative to the crossbridge. These properties were characterized in separate experiments [16,17] and were incorporated in the analysis of the AFD data.

EXPERIMENTAL APPROACH

The skinned muscle fibers from the psoas of rabbits are prepared according to the method of De Beer *et al.* [18]. The single fibers are labeled at the myosin SH1 with 1,5-I-AEDANS (5-iodoacetamidoethylamino-a-naphthalenesulfonic acid) or E5M (eosin-5-maleimide) following the method described by Ludescher and Thomas [7]. It has been reported that, in this way, at least 95% of the probes are bound to SH1 [9].

The muscle fibers are brought into rigor or relaxation by soaking them in a solution with the appropriate concentrations of MgATP and Ca^{2+}. The compositions of these solutions were calculated according to the method described by Fabiato and Fabiato [19]. MOPS [3-(*N*-morpholino)propanesulfonic acid] was used to buffer the solution at a pH of 6.8.

AFD measurements were carried out on a home-built setup similar to that described by Van Gurp *et al.* [13]. The muscle fiber is mounted vertically in the center of the experimental setup in a cylindrical cuvette with a diameter of 2.5 cm filled with buffer. In this way the scattering and refraction of light on the interface of the fiber are dramatically reduced.

RESULTS

AFD was measured on a bundle of five single fibers labeled with either 1,5-I-AEDANS or E5M. The experiments were carried out in rigor and relaxation. Data sets were collected at different excitation wavelengths to utilize the different orientations of the transition dipole moments in the dye molecule.

Distinctly different polarization ratios were obtained from experiments using 1,5-I-AEDANS- and E5M-labeled fibers in the same physiological state. A

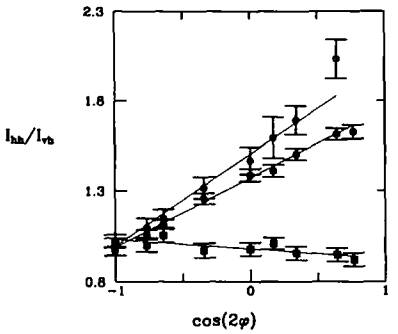

Fig. 1. Depolarization ratios of SH1 labeled muscle fiber. (○) 1.5-I-AEDANS; excitation wavelength, 362 nm. (●) E5M; excitation, 464 nm. (■) E5M; excitation, 337 nm.

change in the wavelength of excitation light also resulted in markedly different polarization ratios (Fig. 1). Since the fibers were in the same physiological state, the differences must be ascribed to the different directions of the transition dipole moments in the system. To extract the order parameters of the crossbridges in the fiber, we have expressed the polarization ratios in terms of three distinct orientations: (i) the orientation of the crossbridge in the fiber, (ii) the orientation of the dye in the crossbridge frame, and (iii) the orientations of the transition dipole moments in the dye frame. A detailed description of the theoretical framework is given in Ref. 20.

The order parameters of the crossbridges were extracted from the experimental data using the nonlinear least-squares Marquart procedure (ZXSSQ) from the IMSL library (Table I). Data sets collected at different excitation wavelengths were analyzed simultaneously in a global target approach. We emphasize that the knowledge of the fourth-rank order parameters is indispensible since the second-rank order parameters are close to zero. The maximum-entropy method may now be invoked for reconstructing the broadest possible distribution function consistent with the experimentally accessible order parameters [21]. Although this choice may not be valid for sharply peaked distributions, we believe that this is the only approach for avoiding any implicit assumptions.

The orientational distribution functions of the crossbridges in rigor and relaxation are sharply peaked at an orientation of about 45° and have a full-width half-maximum of about 20° (Fig. 2). Remarkably, no significant change of orientation is observed between the crossbridges in rigor and relaxation. This observation is at odds with the rotating crossbridge model, which predicts a clear change of orientation. We note here that are experiments are not sensitive to a conformational change of the crossbridge which does not affect the orientation

Table I. The Order Parameters of the Crossbridges in a Muscle Fiber Labeled with 1,5-I-AEDANS and E5M

	Rigor	Relaxation
$\langle P_2 \rangle$	−0.010 ± 0.010	0.000 ± 0.010
$\langle D^2_{02} \rangle$	0.040 ± 0.015	0.000 ± 0.010
$\langle P_4 \rangle$	−0.15 ± 0.05	−0.23 ± 0.05
$\langle D^4_{02} \rangle$	−0.11 ± 0.02	−0.17 ± 0.06
$\langle D^4_{04} \rangle$	0.04 ± 0.06	0.08 ± 0.08

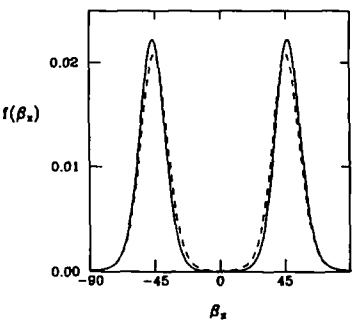

Fig. 2. The orientational distribution function of crossbridges in muscle fiber: (——) rigor; (---) relaxation.

of the binding site of the probes but causes other parts of the crossbridge to undergo a significant rotation. This can be studied following the same approach by labeling other sites on the crossbridge.

CONCLUSION

The results presented here show that the principal features of the orientational distribution of crossbridges in a muscle fiber can be extracted from AFD experiments, in a way independent of the dye molecules and transition dipole moments. Importantly, only a single orientational distribution of the crossbridges is found when the orientations of the dyes relative to the crossbridge and the transition dipole moments in the dye frame are accounted for. This method can be used to map the orientational behavior of the crossbridges at other sites as well. This eventually can lead to a better understanding of the role of crossbridges in the contractile process.

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