USE OF FLUORESCENCE POLARIZATION TO OBSERVE CHANGES IN ATTITUDE OF S-1 MOIETIES IN MUSCLE FIBERS

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ABSTRACT The fluorophore, *N*(iodoacetylamino)-1-naphthylamine-5-sulfonic acid (1,5-IAEDANS), incubated with glycerinated psoas fibers primarily labels the S-1 moieties of such fibers, but it does not impair fiber contractility even when the degree of labeling is as high as 0.8 moles fluorophore per mole myosin. The polarization of the on-axis fluorescence from either the IAEDANS fluorophore, or the intrinsic tryptophane fluorophore, depends on whether the fiber is relaxed, in rigor, or developing isometric tension; furthermore, the changes in polarization on going from one state to another are much the same with either tryptophane or IAEDANS fluorophores. The foregoing is true whether the plane of the exciting light is parallel or perpendicular to the fiber axis. Also, if a fiber is first freed of its myosin by extraction, and is then incubated with IAEDANS-labeled S-1 the resulting polarization approaches that observed with a labeled, unextracted fiber in rigor. By contrast, incubation with the fluorophore, 7-nitro-4-chlorobenz-2-oxa-1,3-diazole (NBD-Cl) confers fluorescence only on actin, without impairing contractility, but the polarization of such fluorescence changes in a different direction and magnitude from myosin-originating fluorescence. It is concluded from these various observations that whether the fluorophore is IAEDANS or tryptophane the polarization change with change in physiological state originates in the S-1 moieties of fibers, and relates to the space attitude of these moieties.

INTRODUCTION

It was shown by Aronson and Morales (1) that the "polarization"[1] [$P = (I_{||} - I_{\perp})/(I_{||} + I_{\perp})$] of the on-axis tryptophane fluorescence emitted by glycerinated psoas fibers excited by plane-polarized light depended on physiological state, i.e., on whether the fiber was relaxed, in rigor, or developing isometric tension. It was suggested that the phenomenon might arise because in the different states the cross-bridges assume different rotational positions. Subsequently, dos Remedios et al. (2) reported some evidence substantiating this interpretation, e.g., the phenomenon occurs also in glycerinated insect fibers and in live frog fibers, and P varies with fiber length in an anticipated way (see below, however); furthermore, tryptophane

[1] The notation $P_{||}$ and $P_{\perp}$ refers to the fluorescence polarization when the excitation plane is, respectively, parallel to and perpendicular to the fiber axis. $I_{||}$ and $I_{\perp}$ are the emitted fluorescence intensities, respectively, parallel to and perpendicular to the excitation plane.

turned out to exist in the S-1 moieties of myosin, but not in the S-2's (the two presumably mobile moieties constituting a cross-bridge). Then, dos Remedios et al. (3), *assuming* the interpretation, found that, by using ATP analogs to "freeze" complexes in the ATPase cycle, they could produce states that appeared to belong in the kinematic cycle of a cross-bridge. Finally, in an interesting "quick-release" experiment, Steiger et al. (4) recorded the polarization changes that woud be anticipated from the behavior of cross-bridges in such an experiment.

The foregoing observations all *suggest* that P is some measure of the rotational attitude of S-1 moieties. Since the measurement of P is essentially instantaneous while the acqusition of an X-ray diffraction pattern is very slow (compared with cycling frequencies of about 100 Hz) it is obviously important to assure the validity of polarization as an attitudinal measurement. In this paper we report results, obtained by a modified procedure, that substantially improve the method and seem to dispel remaining significant doubts about it. In a companion paper (5) we use the improved method to ascertain the behavior of energized S-1 moieties in the absence of actin (at zero filament overlap).

A question about specificity and a question about optics are considered here. (*a*) The use of an "intrinsic probe" like tryptophane is ideal in that it leaves function undisturbed; furthermore, tryptophane exists in only one of the two myosin moieties subject to significant displacement. Nevertheless, actin and the control system also contain tryptophane. Conceivably, conformational changes in these other proteins could generate state-dependent changes in P; in that case P might not be giving information about S-1's at all. What we do to overcome this uncertainty is to use for the measurement of P an extrinsic fluorophore which we are sure attaches to S-1 only. We also show that at least one fluorophore on actin changes with state in a manner different from tryptophane and the S-1 fluorophore. (*b*) In previous work it is only $P_\perp$ (the subscript now referring to the relationship between exciting plane and fiber axis) that has been found to be state-dependent. Because there are many tryptophanes in the system it has been thought that $P_\parallel$ is dominated by fluorophores other than those on S-1. The (S-1)-specific fluorophore, however, can bind to S-1 with at most a 1:1 stoichiometry, and (with the previously-used optics) $P_\parallel$ continues to be state-insensitive. We have traced this anomaly to formerly improper collimation of light by the substage condenser when the $\parallel$-orientation is used; when the collimation is performed by a long focal length lens, or when the light source is an N_2-laser, we can demonstrate state-dependent changes in $P_\parallel$, conjugate to those in $P_\perp$.

EXPERIMENTAL DETAILS

Materials

The dye, *N*(iodoacetylamino)-1-naphthylamine-5-sulfonic acid (1,5-IAEDANS) was synthesized by the method of Hudson and Weber (6), and the dye, 7-nitro-4-chlorobenz-2-oxa-1,3-diazole (NDB-Cl) was synthesized by the method of Boulton et al. (7).

Freshly excised rabbit psoas muscles were tied to glass rods without stretching, and placed into 50% (vol/vol) glycerol-water at 0°C for 6–12 h. The muscles were then transferred to fresh glycerol-water at −15°C, and kept for 3 wk. Before use the muscles were soaked for 1–2 h in 0.1 M KCl + 5 mM Na phosphate, pH 6.8, 0°C. A very thin (diameter, 0.2–0.5 mm) bundle of muscle was now trimmed under a dissecting microscope. Two sets of watchmaker's forceps were used to isolate a single fiber, but the fiber was never touched by the forceps except at its very ends, and it had no damaged segment. The fiber was then transferred to the measuring vessel, and its ends were fastened to two small tin plates (each plate was 3 × 5 mm, and the plates were 5 mm apart). One of these plates was connected by a stainless steel wire to a loudspeaker; thus the fiber could be oscillated at 2 Hz, with an amplitude of about 2% of the fiber length. The other plate was connected to a strain gauge (model UL 2/120, Shinkoh Communication Industry Co., Ltd., Kanagawa-Ken, Japan). The diameter of, and the average sarcomere length in, a single fiber were measured with an eyepiece micrometer disc, calibrated using a stage micrometer. Diameters were in the range of 30–40 μm. Three segments, each 40 μm long, were chosen randomly along the fiber, and the number, n, of sarcomeres in each segment was counted. Fibers were considered acceptable for further study only if the average sarcomere lengths (40 $\mu m/n$) in the three segments agreed within 5%.

Instrumentation

The instrumental arrangement for measuring mechanical parameters and the fluorescence polarization of fibers was basically that previously described (2, 3) except that, (*a*) the excitation bands at 300 nm, 345 nm, and 420 nm were isolated by interference filters (Oriel Optics Corp., Stanford, Conn.) rather than by a monochromator, and (*b*) certain improvements were made in the optical train. The incident light from the 75 W Xe lamp was roughly collimated by a lens attached to the lamp housing. The periphery of the beam was cut off by a 6 mm diaphragm which was anterior to the condensing lens. The Zeiss quartz achromatic condenser originally used was replaced by a 3.8 cm focal length lens (Special Optics, Cedar Grove, N. J.). (*c*) In certain preliminary experiments an N_2-laser (Phase R Corporation, New Durham, N. H.), λ = 337 nm, replaced the Xe source. The ability of the instrument to measure polarization was validated by measuring the fluorescence polarization of NADH dissolved in propylene glycol at 23°C, viz., 0.45. This value is very close to the value reported by Spencer (8), using a conventional polarization fluorometer.

Electrophoresis of extracts through slabs of polyacrylamide gel was accomplished in an Ortec 4200 electrophoresis apparatus (Ortec, Inc., Oak Ridge, Tenn.). After electrophoresis the gels were either stained with Coomassie blue and examined or left unstained and examined visually under a UV lamp (375 nm, max; Gelman Instrument Co., Ann Arbor, Mich.).

Preparation of Labeled Fibers

After the fiber was examined for morphological uniformity, average sarcomere length, average diameter, tension development, dynamic stiffness, and (tryptophane) fluorescence emission, it was tested for its ability to change state. Rigor, relaxation, and active tension development solutions were, respectively, 40 mM KCl, 5 mM $MgCl_2$, 5 mM Na phosphate, 2 mM EGTA; the above plus 5 mM ATP, and the above plus 1.6–2.0 mM $CaCl_2$ ($[Ca^{2+}] \sim 10^{-6} - 10^{-5}$ M), all at pH 7.0, 23°C.

Following the preliminary tests the fiber was either used in an experiment, or it was labeled

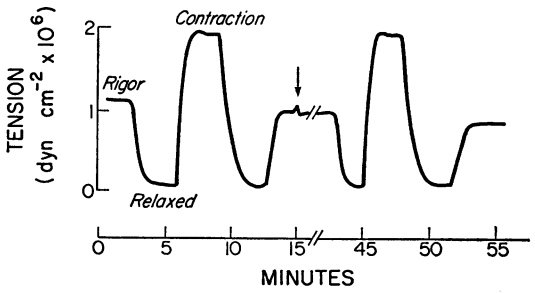

FIGURE 1 Tension development before and after incubation with 5 × 10^{-5} M 1,5-IAEDANS in rigor solution. A single glycerinated fiber was exposed to various (see text) buffer solutions to induce rigor, relaxation, and contraction. At the time indicated by an arrow in the figure, rigor solution containing 1,5-IAEDANS was introduced. After a 27 min incubation at 23°C, relaxing solution was used to wash out unreacted dye.

while mounted at rest length in rigor solution[2] containing 3 to 5 × 10^{-5} M dye for 30–60 min at 22–23°C with occasional stirring. The fiber was then washed to eliminate unbound label and put through a second series of tests to assure that labeling has not impaired function. In the labeled fibers whose behavior is reported here the contractile response was always within experimental error of that exhibited by control fibers. A performance record of a fiber labeled with IAEDANS is shown in Fig. 1. No discernible changes in the long term durability of the fiber were produced by labeling.

The Location of Label

As the polarization of the fluorescence of NBD-Cl showed only small changes with physiological state (see below), extensive analysis of its location on the fiber was not made; however, tests with purified myosin, actin, and the relaxing proteins showed that while NBD-Cl binds with all these proteins, it is only the actin conjugate that is fluorescent (excitation at 420 nm and emission between 500 and 600 nm).

In order to locate IAEDANS, the fiber was shaken gently for 1–2 h in a solution of high ionic strength (9, 10). This procedure reduced the original fiber fluorescence intensity at 480 nm to less than 10% of its original value (of course more intense extraction might reduce the fluorescence further, i.e., it does not follow that the residual 10% arises from nonmyosin proteins). In another experiment, the extract from myofibrils prepared by homogenizing labeled fibers was chromatographed on DEAE cellulose (11). By studying the OD_{340} of the extract and of the tubes that constitute the myosin peak it was ascertained that at least 90% of the fluorescence of the extract arose from myosin, and 5% from the lead peak (which is thought to include C-protein). Another 5% may arise from the trailing peaks which certainly include aggregates of myosin. The myosin fractions were treated with papain. Insoluble papain suspension (0.1 ml) (12) was mixed with 2 ml of 1% myosin in 0.04 M KCl, 2 mM DTT, and 5 mM EDTA (pH 7.0) and was incubated at 23° for 20 min. The amount of papain used split 0.3 μmol of benzoyl-1-arginine ethyl ester

[2] Fibers could also be successfully labeled in relaxation solution, but only if the fiber thus labeled was immediately treated with 2 mM dithiothreitol (DTT); otherwise, its function was impaired. Presumably reaction of the "fast reacting thiol" on S-1 renders a second, critical thiol vulnerable to a deleterious process that is prevented by actin or reversed by DTT (Seidel, J. C., personal communication).

(at 25 mM) per minute at 23°C, pH = 7. After stopping the treatment by centrifugation, electrophoretograms were made on 10% acrylamide gels, of the supernate and of the precipitate. In the electrophoretogram of the supernate, IAEDANS fluorescence was detectable only in the band corresponding to S-1; in those of the precipitate strong fluorescence was observed only in the myosin-HMM band, and faint fluorescence was observed only at a position corresponding to S-1 (we believe that the material is, in fact, S-1 occluded in the precipitate). (This same result was obtained by Mendelson et al. (13) starting with purified and chromatographed myosin.) The moles of 1,5-IAEDANS per mole of myosin were estimated to be between 0.4 and 0.8 in a large number of preparations, assuming that E_{340} = 6,100 M^{-1} cm^{-1} for bound IAEDANS. From the papain digest solution, labeled S-1 was also isolated and purified for use in the "reconstitution" experiment described below.

RESULTS AND DISCUSSION

The results of several experiments in Table I summarize the two objectives attained in this study. IAEDANS, which is bound to the S-1 moieties of the fiber, generates *changes* of $P_\perp$ with state that are identical with those observed using intrinsic tryptophane. (The absolute values of $P_\perp$ given by the two fluorophores are not the same, but this may not be expected, since the optic axes of the two fluorophores almost certainly are differently oriented with respect to the axes of S-1.) Furthermore, $P_\parallel$ changes in a manner that is conjugate to $P_\perp$. As we have explained, the only bound NBD-Cl that can be observed is that on actin. The NBD-Cl fluorophore generates no significant increase in P when the muscle state is changed from rigor to contraction to relaxation. Still another experiment indicates that the polarization effect arises in S-1. The polarization value of an IAEDANS-labeled solution of S-1 at 23°C is P = 0.22. On the other hand, if the myosin of a fiber is extracted by a high ionic strength solution and the "ghost" is then incubated with labeled S-1 (the unbound S-1 being washed off after about 2 h of incubation), the results of five

TABLE I

FLUORESCENCE POLARIZATION FROM SINGLE RABBIT PSOAS FIBERS

State	Tryptophane		IAEDANS		NBD-Cl	
	$P_\perp$	$P_\parallel$	$P_\perp$	$P_\parallel$	$P_\perp$	$P_\parallel$
Rigor	0.132	0.223	0.174 (0.185)	0.288 (0.270)	0.31	0.36
Dev. tension	0.149	0.230	0.191	0.302	0.31	0.36
Relaxation	0.173	0.238	0.210 (0.220)	0.318 (0.298)	0.29	0.34

Values of the polarization of the on-axis fluorescence of single fibers in various physiological states. For tryptophane fluorescence eight different fibers were used, and excitation was at 300 nm; for IAEDANS fluorescence 13 different fibers were used and excitation was at 345 nm; for NBD-Cl fluorescence three different fibers were used and excitation was at 420 nm. For tryptophane and IAEDANS fluorophores the SD of polarizations measured on the same fiber is ±0.002; if the variability among fibers is included (as in this Table) then the SD is ±0.005. For the NBD-Cl fluorophore the SD was ±0.01 if variability among fibers is included. Collimation was accomplished by a UV-transmitting lens of F = 3.8 cm. The numbers in parentheses are single preliminary readings made with a nitrogen laser (λ = 337 nm).

experiments show that now $P_{\perp} = 0.08 \pm 0.02$, and $P_{\parallel} = 0.33 \pm 0.02$. Thus, the attachment of labeled S-1 to a fiber tends to reconstitute a polarizing system similar[3] to that of a directly-labeled fiber.

Even if we grant that the polarization properties of the organized fiber arise in its S-1 moieties, it does not follow that the changes in polarization with state arise because the S-1's have changed in space attitude; for example, contact of S-1 with nucleotide or with actin could produce such conformational changes in S-1 that the fluorophore rotates relative to S-1 even though the S-1 attitude remains constant in space. However, this alternative interpretation is highly improbable. To begin with, we already know from X-ray diffraction work (15–17) that in fact S-1 does change in attitude, at least between rigor and relaxation, and such changes must necessarily alter the polarization properties of (S-1)-bound fluorophores. Secondly, work in progress in our own laboratories has shown that the binding of nucleotide produces no detectable effect on the rotational correlation time of S-1, (i.e., there are no *major* distortions of the S-1 axes), and that the binding of F-actin to S-1 interacting with ATP produces no detectable change in the excited state lifetime of the bound IAEDANS fluorophore (as would be expected if there were a substantial conformational change in the neighborhood of the fluorophore) (Mendelson, R., unpublished results). Finally, we have the result of this work, viz., two different fluorophores, attached at different places, generate closely similar changes in polarization.

We feel that the foregoing results and considerations assure that the polarization, whether measured with intrinsic tryptophane or extrinsic IAEDANS, is a *phenomenological* indicator of S-1 attitude. This is, of course, the only way in which it has been applied hitherto. There remains for the future the interesting problem of developing the suitable theory that will make the polarization an actual angular measurement.

The authors wish to thank Prof. M. F. Morales for his support and helpful advice throughout this work.

Thanks are also due to Dr. R. Haugland for making the dye stuff available, and to Mr. T. Ferrin, Miss K. Ue, and Mrs. S. Putnam for their skillful technical services.

This research has been supported by American Heart Association grant CI-8, National Science Foundation grant GB992X, and U. S. Public Health Service grants HL06285 and HL13649.

Dr. Nihei received support from the Muscular Dystrophy Association and Medical Research Council, Canada.

Dr. Mendelson is a Special Fellow of the U. S. Public Health Service.

Received for publication 21 August 1973.

[3] As in the analogous X-ray diffraction experiment carried out by Rome (14), it is not at all obvious that an *identical* polarizing system should be reconstituted, for S-1's "decorating" actin filaments may not be in the same lattice as are the actin-linked S-1 moieties of myosin molecules of a thick filament.

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