Romey, G., Abita, J. P., Schweitz, H., Wunderer, G., & Lazdunski, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4055–4059.
Scheffler, J.-J., Tsugita, A., Linden, G., Schweitz, H., & Lazdunski, M. (1982) *Biochem. Biophys. Res. Commun. 107*, 272–278.
Schweitz, H., Bidard, J.-N., Frelin, C., Pauron, D., Vijverberg, H. P. M., Mahassneh, D. M., & Lazdunski, M. (1985) *Biochemistry 24*, 3554–3561.
Smith, C. D., DeLucas, L., Ealick, S. E., Schweitz, H., Lazdunski, M., & Bugg, C. E. (1984) *J. Biol. Chem. 259*, 8010–8011.
Tanaka, M., Haniu, M., Yasunobu, K. T., & Norton, T. R. (1977) *Biochemistry 16*, 204–208.
Wemmer, D. E., Kumar, N. V., Metrione, R. M., Lazdunski, M., Drobny, G., & Kallenbach, N. R. (1986) *Biochemistry 25*, 6842–6849.
Wider, G., Macura, S., Kumar, A., Ernst, R. R., & Wüthrich, K. (1984) *J. Magn. Reson. 56*, 207–234.
Widmer, H., Wagner, G., Schweitz, H., Lazdunski, M., & Wüthrich, K. (1988) *Eur. J. Biochem. 171*, 177–192.
Wunderer, G. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1193–1201.
Wunderer, G., & Eulitz, M. (1978) *Eur. J. Biochem. 89*, 11–17.
Wunderer, G., Fritz, H., Wachter, E., & Machleidt, W. (1976) *Eur. J. Biochem. 68*, 193–198.
Wüthrich, K. (1983) *Biopolymers 22*, 131–138.
Wüthrich, K., Wider, G., Wagner, G., & Braun, W. (1982) *J. Mol. Biol. 155*, 311–319.
Wüthrich, K., Billeter, M., & Braun, W. (1984) *J. Mol. Biol. 180*, 715–740.
Zykova, T. A., Vinokurov, L. M., Kozlovskaya, E. P., & Elyakov, G. B. (1986) *Bioorg. Khim. 11*, 159–165.

Fluorescent Modification and Orientation of Myosin Sulfhydryl 2 in Skeletal Muscle Fibers†

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ABSTRACT: We describe a protocol for the selective covalent labeling of the sulfhydryl 2 (SH2) on the myosin cross-bridge in glycerinated muscle fibers using the sulfhydryl-selective label 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD). The protocol promotes the specificity of IANBD by using the ability to protect sulfhydryl 1 (SH1) from modification by binding the cross-bridge to the actin filament and using cross-bridge-bound MgADP to promote the accessibility of SH2. We determined the specificity of the probe using fluorescence gel scanning of fiber-extracted proteins to isolate the probe on myosin subfragment 1 (S1), limited proteolysis of the purified S1 to isolate the probe on the 20-kilodalton fragment of S1, and titration of the free SH1's on purified S1 using the radiolabeled SH1-specific reagent [^{14}C]iodoacetamide or enzymatic activity measurements. We estimated the distribution of the IANBD on the fiber proteins to be ~77% on SH2, ~5% on SH1, and ~18% on troponin I. We characterized the angular distribution of the IANBD on cross-bridges in fibers when the fibers are in rigor, in relaxation, in the presence of MgADP, and in isometric contraction using wavelength-dependent fluorescence polarization [Ajtai, K., & Burghardt, T. P. (1987) *Biochemistry 26*, 4517–4523]. With wavelength-dependent fluorescence polarization we use the ability to rotate the transition dipole in the molecular frame using excitation wavelength variation to investigate the three angular degrees of freedom of the cross-bridge. We find that the SH2 probe distinguishes the different states of the fiber such that rigor and MgADP are ordered and maintain a similar orientation throughout the excitation wavelength domain. The relaxed cross-bridge is ordered and has an orientation that is distinct from the orientation of the cross-bridge in rigor and MgADP over the entire wavelength domain. The active isometric cross-bridge is also ordered and has a distinctive polarization spectrum that has a different shape from all of the other states measured. The active isometric cross-bridge is also oriented differently from the other states, suggesting the presence of a predominant actin-bound cross-bridge state that precedes the power stroke during muscle contraction.

Motions of protein elements of the contractile apparatus in skeletal muscle are often studied by the specific modification of reactive side chains on the protein elements. The fast-reacting thiol, sulfhydryl 1 (SH1), of the myosin cross-bridge was specifically modified by a variety of fluorescent and electron spin resonance (ESR) probes and its orientation studied for many years (Nihei et al., 1974; Borejdo & Putnam, 1977; Thomas & Cooke, 1980; Borejo et al., 1982; Ajtai & Burghardt, 1986). Although the conclusion remains somewhat controversial, it is generally accepted that when myosin is bound to actin the SH1 thiol maintains more than one orientation relative to the actin filament in a muscle fiber. The angular disposition of other points on the myosin cross-bridge was less thoroughly studied, but there is some noteworthy data. The nucleotide binding site on the myosin cross-bridge was noncovalently probed with fluorescent (Yanagida, 1981, 1985) and spin analogues (Crowder & Cooke, 1987). These studies

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agree that stretching the fiber does not induce a change in the orientation of the nucleotide site probe.

The implications of the probe studies of cross-bridge orientation are fundamental in view of the present models of muscle contraction. The rotating cross-bridge model of contraction holds that the cross-bridge rotates while attached to actin to produce muscle shortening against a load (Huxley, 1969; Huxley & Simmons, 1971; Huxley & Kress, 1985). The observation that not all probes (even those probes on the same side chains of myosin) detect a significant displacement of the cross-bridge motivated us to develop more elaborate formal (Burghardt, 1984; Burghardt & Thompson, 1985) and experimental (Ajtai & Burghardt, 1987) approaches to the problem of ascertaining what probes tell us about the angular disposition of the cross-bridges.

The orientation of a fluorescent probe within protein elements of a biological assembly is crucial in the determination of angular movement of the protein element (Ajtai & Burghardt, 1987). If the probe is oriented such that its transition dipole is aligned with the axis of rotation of the protein element, then the probe will report no angular displacement of the element. Fluorescence polarization spectroscopy, where the fluorescence polarization is measured as a function of the excitation wavelength, gives us the capability of rotating the absorption dipole of the probe inside the molecular frame of the protein element while simultaneously monitoring probe orientation. With this method we remove this particular ambiguity in the fluorescence polarization signal due to the vectoral nature of the dipolar transition.

A second useful tactic of ascertaining cross-bridge orientation during muscle contraction is to probe a variety of sites on the cross-bridge. The combination of data from probes of different side chains of the cross-bridge provides a more detailed picture of the cross-bridge angular distribution, provided the cross-bridge is not a rigid body. We used the technique of fluorescence polarization spectroscopy on a probe of the cross-bridge located at a new labeling site. We describe here our findings.

We achieved specific covalent modification of the "second thiol" on the myosin cross-bridge, sulfhydryl 2 (SH2), with the probe 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD). The labeling procedure takes advantage of the increased reactivity of SH2 in the presence of MgADP (Sekine & Yamaguchi, 1963; Yamaguchi & Sekine, 1966; Sekine & Kielley, 1969; Kameyama et al., 1977) and the decreased reactivity of SH1 in the presence of actin (Duke et al., 1976) by labeling the fiber in the MgADP state where the cross-bridges bind both actin and MgADP. We verified the specificity of the probe by first localizing the probe on the 20-kilodalton (kDa) fragment of myosin subfragment 1 (S1) containing SH1 and SH2 and then titrating the SH1 of IANBD-modified S1 with the SH1-specific reagent [^{14}C]iodoacetamide to show that nearly 100% of the SH1's were unmodified by the IANBD. In the muscle fiber we estimate that ~77% of the IANBD is on SH2, ~5% is on SH1, and ~18% is on troponin (TnI).

We established the ability of the modified fibers to contract by comparing the active isometric tension generated by unmodified and SH2-modified glycerinated fibers. When as much as 40% of the SH2's in a fiber were modified with IANBD, the active isometric tension of the fiber was identical with that of a native fiber.

We measured the fluorescence polarization excitation spectrum from the IANBD-labeled fibers when the fibers are in rigor, in relaxation, in the presence of MgADP, and in isometric contraction. Our results show that the SH2 side chains maintain distinctive orientation distributions such that fibers in rigor and in the presence of MgADP have a similar cross-bridge orientation that is distinct from the active-state orientation. We also found that the relaxed cross-bridge orientation distribution differed from all of the other distributions measured.

Control experiments indicated that the fluorescence emission spectrum from IANBD-labeled fibers in rigor, in relaxation, in the presence of MgADP, and in contraction was unaltered by the binding of a nucleotide and/or actin. Because the emission spectrum is sensitive to the local environment of the probe, we interpret these results as indicating that the local environment of the SH2-bound probe was not altered in the different fiber states. This interpretation is consistent with the view that changes in the probe orientation reflect changes in the orientation of the myosin cross-bridge. We conclude that the results showing a different probe orientation for the cross-bridge in rigor and MgADP states compared to active isometric fibers indicate that the cross-bridge also maintains a different orientation in the active isometric state compared to the rigor and MgADP states.

Materials and Methods

Chemicals. ATP, trypsin, and α-chymotrypsin were from Sigma (St. Louis, MO). ADP was from Calbiochem (La Jolla, CA). [^{14}C]Iodoacetamide ([^{14}C]IAA), with a specific activity of 53 mCi/mmol, was from Amersham (Arlington Heights, IL). 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD), 5-[[[(iodoacetyl)amino]-ethyl]amino]naphthalene-1-sulfonic acid (1,5-IAEDANS), and tetramethylrhodamine-5(and 6)iodoacetamide (IATR) were purchased from Molecular Probes (Eugene, OR). All chemicals were of analytical grade.

Solutions. Rigor solution contained 80 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 5 mM potassium phosphate, and 1 mM DTT at pH 7. Relaxing solution was rigor solution with 4 mM MgATP. MgADP-containing solution was rigor solution with 4 mM MgADP. The activating solution has the same composition as relaxing solution except EGTA was replaced with 0.1 mM $CaCl_2$. Rigor + Ca solution has the same composition as rigor solution except that EGTA was replaced with 0.1 mM $CaCl_2$.

Preparation and Labeling of Muscle Fibers. We obtained rabbit psoas muscle fibers as described previously (Borejdo et al., 1979) and stored them in relaxing solution containing 50% (v/v) glycerol at −15 °C for up to several weeks. The preparation of fibers for labeling was also done as previously described (Burghardt et al., 1983). We labeled the skinned fibers with 125 μM IANBD in MgADP solution without DTT for 15 min at 4 °C with intensive stirring. The reaction was stopped with 1 mM DTT, and the excess of the dye was washed out with relaxing solution. Estimation of the labeling intensity and specificity was carried out either on the homogenate of the whole fiber or on purified proteins isolated from the fiber. In order to study the target protein of our labeling procedure, whole labeled fibers were homogenized and incubated in SDS sample buffer (Laemmli, 1977) with stirring for 2 h at room temperature. The homogenate was pelleted in a centrifuge, and the supernatant was applied on a 15% SDS–PAGE slab gel. The electrophoretogram was analyzed either by visualization of the labeled proteins by fluorescence or by staining the gel electrophoretogram with Coomassie brilliant blue. The parallel analysis of the two pictures served as a comparative analysis for the identification of the labeled proteins or protein fragments. The quantitative distribution

of the dye was measured by scanning the fluorescence of the unstained electrophoretogram. The more detailed specificity studies were done on myosin and S1 purified from the labeled fibers.

The labeling of control fibers with 1,5-IAEDANS and IATR was performed according to Borejdo and Putnam (1977) and Borejdo et al. (1979), respectively.

Proteins. Myosin was prepared from labeled glycerinated muscle fibers according to the method of Crowder and Cook (1984) with slight modification. The fibers were first washed with 5 mM Tris-HCl with 1 mM EGTA, at pH 8, and subsequently homogenized in the myosin-extracting solution containing 0.6 NaCl, 50 mM Tris-HCl, 5 mM sodium pyrophosphate, 5 mM $MgCl_2$, and 1 mM EGTA at pH 8. The extraction was carried out in ice for 3 h with stirring. The homogenate was separated by centrifugation, and the supernatant containing the extracted myosin was collected. Myosin was purified by low ionic strength precipitation, and subsequently S1 was prepared according to the procedure of Weeds and Taylor (1975). The fluorescence dye incorporation was estimated from the absorption spectra of the labeled proteins. Myosin and S1 concentrations were calculated by using absorption coefficients at 280 nm of A(myosin) = 0.54 and A(S1) = 0.77 for 1 mg/mL protein concentration and 1-cm path length. Bound IANBD was determined by using the molar extinction coefficient at 495 nm of $\epsilon = 2.7 \times 10^4$ M^{-1} cm^{-1}. The molecular masses of myosin and S1 were estimated to be 450 and 115 kDa, respectively.

ATPase Activity Measurement. The Ca^{2+}- or K^+-EDTA-activated ATPase activity was measured by using the modified Fiske and SubbaRow phosphate determination method of Bârány et al. (1967). The K^+-EDTA-ATPase activity was measured in 0.6 M KCl, 6 mM EDTA, and 25 mM Tris-HCl at pH 8 and 25 °C. The Ca^{2+}-activated ATPase was measured in 0.6 M KCl, 5 mM $CaCl_2$, and 25 mM Tris-HCl at pH 8 and 30 °C. The protein concentration of the assay was 0.02–0.03 mg/mL, and ATP concentration was 2.0 mM.

Tryptic Digestion of S1. Labeled and unlabeled S1 was fragmented with trypsin according to Bâlint et al. (1975, 1978). The digestion was carried out in 50 mM Tris-HCl at pH 8 and 25 °C with a S1:trypsin ratio of 1:80 (w/w). The reaction was stopped with the addition of soybean trypsin inhibitor with a ratio 1:3 (w/w) of trypsin:inhibitor.

The peptide composition of the digest was analyzed by SDS–PAGE according to Laemmli (1970). The acrylamide concentration of the gel was 15%. Gels were analyzed for fluorescence after finishing the electrophoresis and for protein composition by staining the peptides with Coomassie brilliant blue.

Determination of the Free SH1 Groups of Fiber S1. Unlabeled S1 and labeled S1 originating from fibers were incubated with a 20–40-fold molar excess of [^{14}C]IAA in 50 mM TES at pH 7. Under these conditions the iodoacetamide reacts exclusively with the SH1 groups of the S1 molecule (Takashi et al., 1976; Bâlint et al., 1978). The reaction was stopped by the addition of 16% trichloroacetic acid, and the precipitate was collected by filtration through a Whatman glass microfiber filter (GF/F) and washed with several volumes of 5% trichloroacetic acid. The filter containing a known amount of precipitated protein with the covalently attached radiolabeled SH1 probe was dropped into a scintillation vial containing Safety Solve scintillation cocktail (Research Products International Corp., Mount Prospect, IL) and measured for scintillation counts. Calculation of the accessible SH groups was based on the [^{14}C]IAA incorporation related to the molar concentration of S1. Parallel studies of the SH1 modification were performed by using ATPase measurements before and after the radioactive labeling.

Quantitative Fluorescence Measurements. The spectroscopic measurements were performed on a SLM 8000 spectrofluorometer (SLM Instruments, Urbana, IL) equipped with Glan–Thompson polarizers. A 1.4 × 3.0 cm rectangular stainless steel support, made to fit inside a standard fluorescence cuvette, held mounted fibers in the excitation beam path (Burghardt & Ajtai, 1985). The emission was collected at 90° from the excitation beam path. The emission wavelength was selected with a band-pass filter transmitting a wavelength width of 40 nm centered at λ_{em} = 550 nm. We oriented the fiber such that the fiber axis was perpendicular to the excitation and collected emission beams.

An experiment on muscle fibers consisted of the collection of the fluorescence intensities $F_{\perp,\perp}$ and $F_{\perp,\parallel}$ from the IANBD-modified fibers as a function of the excitation wavelength. The first index on F corresponds to the direction of the linear polarization of the excitation beam and the second to the linear polarization of the emitted light. The symbols $\parallel$ and $\perp$ indicate directions relative to the fiber axis. These quantities have an identical but arbitrary normalization so that they are combined in the ratio

$$P_\perp = (F_{\perp,\perp} - F_{\perp,\parallel})/(F_{\perp,\perp} + F_{\perp,\parallel}) \quad (1)$$

We summarize our data by plotting $P_\perp$ as a function of λ_{ex}. $P_\perp = 0$ for all λ_{ex} for a random distribution of probes, and when $P_\perp$ changes sign a probe rotation is unambiguously signaled.

Emission spectra of the IANBD-labeled fibers were recorded on the same instrument in the emission wavelength scanning mode. The excitation wavelength in these experiments was fixed at λ_{ex} = 420 nm, where all of the states of the muscle fiber have similar $P_\perp$'s. The emission spectra were recorded of the intensity $F_{\perp,\perp}$ over the range of emission wavelengths 400 nm $\leq \lambda_{em} \leq$ 600 nm. We measured $F_{\perp,\perp}(\lambda_{em})$ for fibers in rigor, in relaxation, in the presence of MgADP, and in contraction and observed identical spectra for these states. These data indicate no change in the local environment of the probe as a result of the binding of a nucleotide and/or actin.

All fluorescence measurements were performed at 6–8 °C.

Fiber Tension Measurements. Single labeled and unlabeled fibers were mounted between a fixed support and a flexible stainless steel rod of diameter 0.003 in. The single fibers were bathed in rigor, relaxation, or contracting solution. The flexible rod was reversibly displaced from a zero-point tension by fiber tension as observed in a dissecting microscope with a graduated scale in the eyepiece superpositioned on the fiber image. Fibers were mounted in rigor and then activated by contracting solution, and the displacement of the wire was recorded as a function of time. Maximum active tension was observed within 0.5–1.0 min. The fibers were then washed with relaxing solution to verify that the active tension was reversible. We compared the maximum tension generated in labeled and unlabeled fibers in arbitrary units of wire displacement over fiber cross-sectional area.

Results

Evidence for Specific Labeling of SH2 on Cross-Bridges. Figure 1 indicates the localization of IANBD and 1,5-IAEDANS in labeled muscle fiber proteins using SDS–PAGE of the fiber extract. Panel a shows the Coomassie-stained gel pattern of the same gel visualized with fluorescence in panel b. Lane 9 shows the IANBD is predominantly localized in

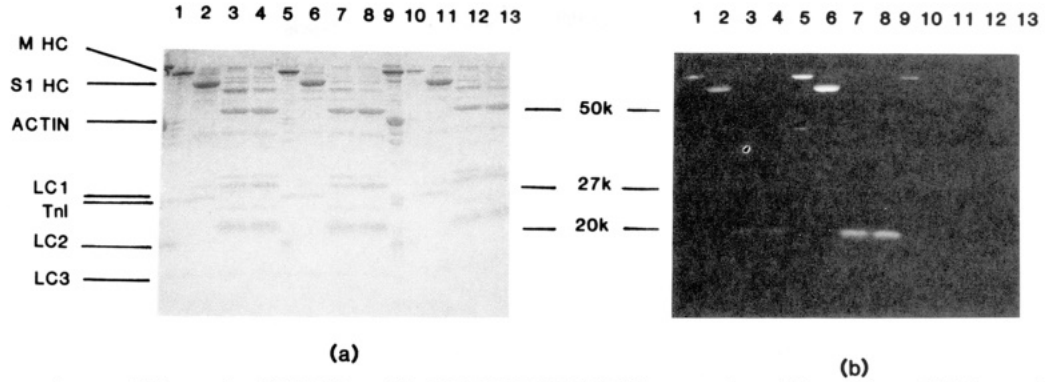

FIGURE 1: Comparative specificity study of IANBD- and 1,5-IAEDANS-labeled fiber proteins. Abbreviations: MHC, myosin heavy chain; S1 HC, S1 heavy chain; LC, light chain; and TnI, troponin I. Panel a: Coomassie-stained SDS–PAGE of muscle fiber proteins. Lanes: (1) Myosin extracted from IANBD-labeled fibers; (2) S1 from IANBD-labeled fiber myosin; (3) tryptic digest of IANBD–S1 (digestion time 15 min); (4) tryptic digest of IANBD–S1 (digestion time 30 min); (5) myosin extracted from 1,5-IAEDANS-labeled fibers; (6) S1 from 1,5-IAEDANS-labeled fiber myosin; (7) tryptic digest of 1,5-IAEDANS–S1 (digestion time 15 min); (8) tryptic digest of 1,5-IAEDANS–S1 (digestion time 30 min); (9) protein extract of IANBD-labeled fiber; (10) myosin extract of unlabeled fiber; (11) S1 from unlabeled fiber myosin; (12) tryptic digest of unlabeled S1 (digestion time 15 min); (13) tryptic digest of unlabeled S1 (digestion time 30 min). Panel b: same as panel a under UV illumination. Lanes 3 and 4 compared with lanes 7 and 8 show that IANBD migrates with the 20-kDa fragment of S1 as does 1,5-IAEDANS. Comparison of lanes 12 and 13 with lanes 3 and 4, or 7 and 8, indicates that the presence of the covalent probe does not change the digestion profile of S1.

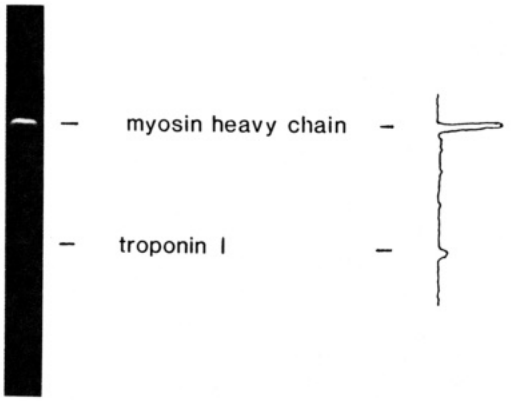

FIGURE 2: Quantitative distribution and localization of IANBD incorporation in muscle fiber proteins. SDS–PAGE and fluorescence scan of fiber extract indicating 82% of the intensity in the myosin heavy chain and 18% in troponin I.

the heavy chain of myosin with some labeling of troponin I. With myosin and S1 purified from the labeled fibers, IANBD is on the heavy chain fragment of myosin (lanes 1 and 2) as is the SH1-specific probe 1,5-IAEDANS (lanes 5 and 6). Limited tryptic proteolysis of the S1 generated a fluorescent 20-kDa fragment for both IANBD (lanes 3 and 4) and 1,5-IAEDANS (lanes 7 and 8), proving that the label modified one of the reactive SH's (Bálint et al., 1978). The Coomassie-stained digestion pattern of the myosin heavy chain after fluorescent modification with IANBD and 1,5-IAEDANS was identical with that of the unmodified myosin heavy chain (lanes 10–13) and characteristic of proteolysis in the absence of nucleotide.

Figure 2 shows the quantitative distribution and localization of IANBD in muscle fiber proteins using SDS–PAGE of the fiber extract. We see that the IANBD is predominantly localized in the heavy chain of myosin with some labeling of troponin I (see also Figure 1, panels a and b, lane 9). The fluorescence intensity scan of the gel lane indicates that ~82% of the intensity is in the myosin heavy chain band while the remaining 18% is in the troponin I band. It has been shown that modification of troponin I with IANBD does not affect the actomyosin interaction or Ca^{2+} sensitivity of the reconstituted actomyosin system (Green, 1986).

The fluorescent label distributions in extracted muscle proteins, resulting from the fluorescence scanning of gel lanes as in Figure 2, are summarized in Table I for 1,5-IAEDANS [from Borejdo and Putnam (1977)], IATR (data not shown), and IANBD (from Figure 2). Table I shows that the total amount of nonspecific IANBD in a fiber is very similar to the total amount of nonspecific probe from the SH1-directed probes 1,5-IAEDANS and IATR. Unlike the SH1-directed probes, all of the nonspecific IANBD resides on troponin I.

Table I: Fluorescent Label Distributions in Muscle Proteins

label	myosin HC[a]	actin	LC1[a]	TnI[a]
1,5-IAEDANS[b]	0.86	0.09	0.0	0.05
IATR	0.85	0.08	0.07	0.0
IANBD	0.82	0.0	0.0	0.18

[a] Abbreviations: HC, heavy chain; LC, light chain; TnI, troponin I. [b] Data taken from Borejdo and Putnam (1977).

Table II: ATPase Activity of Myosin Extracted from Labeled Fibers

label	label stoichiometry (mol of dye/mol of cross-bridge)	ATPase act. (% rel to unlabeled myosin)[a]		contractility (% rel to unlabeled fiber)
		K^+-EDTA	Ca^{2+}	
IATR	0.30	76.0	180.8	100
1,5-IAEDANS	0.47	49.3	343.9	100
IANBD[b]	0.42	57.5	82.3	12
IANDB[c]	0.36	86.0	100.5	100 ± 27[d]
IANBD[e]	1.0	25.0	68.5	

[a] Unlabeled myosin Ca^{2+}-ATPase activity = 0.366 μmol of phosphate/(mg of protein·min); K^+-EDTA-ATPase activity = 2.35 μmol of phosphate/(mg of protein·min). [b] Fiber labeled in relaxation. [c] Fiber labeled in attached state in the presence of MgADP. [d] Standard error in the mean with six independent filter preparations. [e] Purified S1 treated with IANBD without nucleotide in 50 mM TES at pH 7.0.

Table II shows representative ATPase activities and labeling stoichiometry from myosin extracted from fibers for the probes IATR, 1,5-IAEDANS, and IANBD. Three different labeling conditions are demonstrated for IANBD. Both the SH1-specific probes IATR and 1,5-IAEDANS showed elevated Ca^{2+}-ATPase and reduced K^+-EDTA-ATPase relative to unmodified myosin. For these probes the reduction of the K^+-EDTA-ATPase is proportional to the degree of cross-bridge modification. The data for IATR and 1,5-IAEDANS are indicative of specific SH1 modification in the fibers. ATPase activity from myosin modified by IANBD shows that the site of IANBD modification is dependent on the condition

Table III: SH Titration and ATPase Activity of S1 from Labeled Fibers[a]

	label stoichiometry (mol of probe/mol of S1)		ATPase act.	
	fluorescent	[^{14}C]IAA	Ca^{2+}	K^+-EDTA
unlabeled	0.0	1.03 ± 0.09	676 ± 2	9 ± 1
1,5-IAEDANS	0.24 ± 0.04	0.0	296 ± 5	82 ± 2
		0.82 ± 0.06	687 ± 18	5 ± 2
IANBD	0.33 ● 0.04	0.0	104 ± 8	89 ± 5
		0.96 ± 0.1	494 ± 19	9 ± 2

[a] There were seven different S1 preparations. The ATPase activities for unlabeled S1 were as follows: for K^+-EDTA, 4.82 ● 0.02 μmol of phosphate/(mg of S1·min); and for Ca^{2+}, 0.62 ± 0.03 μmol of phosphate/(mg of S1·min).

of the fiber during modification. When IANBD labeling is done in intact fibers in relaxation, both ATPase activities are reduced, indicative of the modification of both SH1 and SH2 (Sekine & Yamaguchi, 1963). If the labeling is done on fibers in the presence of MgADP, Ca^{2+}-ATPase is unaffected and K^+-EDTA-ATPase is slightly reduced. The changes in the ATPase activities are not consistent with the probe modification of SH1 and are very similar to changes observed after the specific modification of SH2 (Reisler et al., 1974), suggesting that IANBD specifically modifies SH2. When purified S1 is modified in TES buffer without nucleotide, again both ATPases are reduced, indicative of the modification of both SH1 and SH2, showing an affinity of the probe for SH2 in contradiction to the results of Miki and Wahl (1984).

The usual demonstration of specific labeling of SH2 using the hydroxylamine cut of 20 kDa that separates SH1 from SH2 (Sutoh, 1981) is not possible with IANBD due to the instability of the probe to the extreme pH and temperature conditions of the hydroxylamine treatment. We found that the chromophore was cleaved from myosin by hydroxylamine. Due to this complication we tried an indirect approach where we titrated the free SH1's of purified S1 from IANBD-treated fibers using the radiolabeled SH1-specific reagent [^{14}C]IAA (Takashi et al., 1976; Bâlint et al., 1978).

Table III shows the results of the titration of SH1 with [^{14}C]IAA. Unlabeled S1, 1,5-IAEDANS–S1, and IANBD–S1 prepared from fibers were labeled with [^{14}C]IAA as described under Materials and Methods. As shown previously, we found that the unlabeled S1 had a one-to-one stoichiometry with [^{14}C]IAA (Takashi et al., 1976). The measurement of Ca^{2+}- and K^+-EDTA-ATPase from this S1 confirms that [^{14}C]IAA is very nearly 100% specific for the SH1 thiol. 1,5-IAEDANS–S1, with a mole-to-mole ratio of fluorescent probe to protein of 0.24 ± 0.04, was further modified with [^{14}C]IAA and showed a mole-to-mole ratio of radiolabel to protein of 0.82 ± 0.06. The Ca^{2+}- and K^+-EDTA-ATPases confirm that only SH1 was modified in the doubly reacted S1. IANBD–S1, with a mole-to-mole ratio of fluorescent probe to protein of 0.33 ± 0.04, was further modified with [^{14}C]IAA and showed a mole-to-mole ratio of radiolabel to protein of 0.96 ± 0.10. This result suggested that on the average 96% of the SH1's were unmodified after treatment with IANBD even though 33% of the S1's were labeled with IANBD. This result, together with evidence presented above showing that IANBD is localized in the 20-kDa fragment of modified S1 from labeled fibers, suggests that IANBD modifies predominantly the SH2 thiol when labeling is done by our protocol. Given that, according to Figure 2, ~82% of the IANBD fluoresence intensity is localized within the myosin heavy chain, then the results of the titration of SH1 with [^{14}C]IAA suggest that, of the 82% on the heavy chain, ~77% is from SH2 labeling, and ~5% is from SH1 labeling.

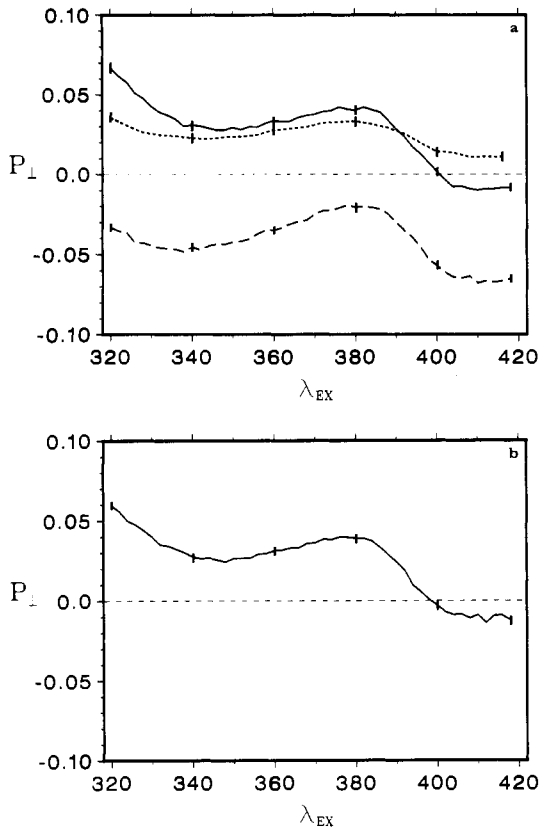

FIGURE 3: $P_\perp$ as a function of excitation wavelength, λ_{ex}, in nanometers measured from IANBD-labeled fibers in rigor (—), in relaxation (---), and in active isometric contraction (···) (a) and in the presence of the MgADP (b). The curves have 2-nm resolution. The vertical error bars indicate standard error in the mean with n = 4–13 different fiber preparations.

Further evidence of the specific modification of SH2 by IANBD is found in the results from the ATPase measurements for IANBD–S1 and S1 modified by both IANBD and [^{14}C]IAA summarized in Table III. We see that both the Ca^{2+}- and K^+-EDTA-ATPases are nearly unaffected by modification with IANBD alone. When IANBD–S1 is further modified by [^{14}C]IAA, the ATPases are changed in a manner characteristic of SH1 and SH2 labeling; i.e., the K^+-EDTA-ATPase activity decreased linearly with the modification of SH1, and the Ca^{2+}-ATPase is inhibited relative to the maximally activated Ca^{2+}-ATPase.

Fiber Contractility Measurements. We judged the contractility of the fiber by measuring the maximum force per unit cross-sectional area generated in isometric contraction by labeled and unlabeled fibers. The results summarized in Table II showed that the SH1-directed probes did not affect the contractility of the fibers when as many as half of the cross-bridges were modified. The result confirms earlier work done on SH1-directed probes (Crowder & Cooke, 1984). Fibers modified by IANBD in the presence of MgADP showed no inhibition of the contractility. The contractility of fibers modified by IANBD in relaxation was drastically reduced. This reduction in contractility was not linearly related to the fraction of modified cross-bridges and implies that the probe must be inducing a cooperative effect to inhibit contraction.

Fluorescence Polarization Spectroscopy from Labels at SH2. Shown in Figure 3 are $P_\perp$ excitation spectra (see eq 1) from IANBD-labeled fibers in rigor, in relaxation, in the presence of MgADP, and in active isometric contraction. These data show that cross-bridges in rigor and in the presence of MgADP maintain similar attitudes throughout the probe

transition dipole orientations achieved in the excitation scan. The relaxed cross-bridges are ordered and have an orientation distinct from the other states. The $P_\perp$ excitation spectrum for probes on active cross-bridges is qualitatively different from those for rigor and MgADP over nearly all of the excitation scan. In the wavelength domain 400 nm $\leq \lambda_{ex} \leq$ 420 nm, the sign of $P_\perp$ is positive for active isometric fibers but negative for fibers in rigor, in relaxation, and in the presence of MgADP. This result indicates conclusively that probes of SH2 on active fiber cross-bridges are oriented different from the other states of the fiber measured.

The emission spectra of IANBD-labeled fibers were observed for fibers in rigor, in relaxation, in the presence of MgADP, and in active isometric contraction (data not shown). The spectra were identical in each case, indicating that the local environment of the probe is unaffected by the binding of nucleotides and/or actin to the cross-bridges. We interpret these data as suggesting that the probe maintains a constant orientation relative to the cross-bridge for all the states of the fiber investigated.

Effect of IANBD–TnI on $P_\perp$. The excitation spectrum and absolute emission intensity from IANBD-labeled fibers in rigor were measured in the presence and absence of Ca^{2+} and exogenous S1 to examine the effect of IANBD–TnI on $P_\perp$. It was shown previously that the presence of Ca^{2+} and S1 perturbs the conformation of TnI such that when TnI is specifically labeled with IANBD, large fluorescence intensity changes occur upon addition of Ca^{2+} or S1 (Trybus & Taylor, 1980; Rosenfeld & Taylor, 1985; Green, 1986). We found that $P_\perp$ from IANBD-labeled fibers in rigor was unchanged when saturating S1 or rigor + Ca solution was added. We also noted that the absolute intensity of the fluorescent emission was unchanged under these conditions. These data suggest that the IANBD on TnI does not contribute significantly to $P_\perp$ or to the fluorescent intensity.

Discussion

We describe a protocol for the specific covalent labeling of the SH2 thiol of the myosin cross-bridge in glycerinated muscle fibers with the fluorescent probe IANBD. We estimate from our specificity studies that the probe is approximately as specific for SH2 as IATR and 1,5-IAEDANS are for SH1. We also show that when the extent of the labeling in the fiber is as much as 0.4 mol of IANBD/mol of cross-bridge, the maximum isometric force generated is not diminished by the presence of the label.

The covalent labeling of different sites on the cross-bridge is an important research objective in that presently SH1 is the only other site that can be covalently and specifically modified in a fiber. Clearly it is important that we test the conclusions drawn from studies of probes on SH1 and elsewhere by making similar studies of SH2. We have begun the characterization of SH2-bound IANBD using fluorescence polarization spectroscopy to determine cross-bridge orientation changes when the physiological state of the fiber is altered.

Whether the actin-bound myosin cross-bridge can maintain more than one orientation relative to the actin filament is a question we wish to answer with our probe investigations. We have introduced certain theoretical and experimental methods to assist us in answering this question. Originally we developed a model-independent theory to unify the treatment of data from the probe techniques and to establish a model-independent criterion for discerning a probe rotation from effects causing probe disordering. In particular, we showed that when the polarization ratio $P_\perp$ (see eq 1), measured from an ordered assembly of probes, changes sign, then the probe has certainly undergone a rotation (Ajtai & Burghardt, 1987). This sign change cannot be interpreted as an increase or decrease in the random portion of the probe angular distribution.

Recently we pointed out the advantages of measuring $P_\perp$ as a function of excitation wavelength (Ajtai & Burghardt, 1987). With excitation wavelength variation we can cause the transition dipole of the probe to change its orientation relative to a molecular frame fixed in the protein element to which the probe is attached. In this way we can probe differing angular degrees of freedom of the protein element. In application to cross-bridges in muscle fibers, we measure $P_\perp$ as a function of the excitation wavelength, giving a definitive answer to the question of whether or not the cross-bridge can maintain more than one orientation relative to the actin filament. We found that both of the SH1-bound fluorescent probes, 1,5-IAEDANS and IATR, showed that the cross-bridge maintains more than one orientation when bound to actin (Ajtai & Burghardt, 1986, 1987).

It is reasonable to measure $P_\perp$ as a function of excitation wavelength from a probe on a new labeling site on the cross-bridge to solidify our view that the cross-bridge can maintain various attitudes relative to the actin filament. What we report here on the SH2 label supports the conclusion that the actin-bound cross-bridge can maintain more than one orientation relative to the actin filament.

The SH1 probe IATR and the SH2 probe IANBD distinguish the orientation of the active isometric state of fibers from that present in rigor and MgADP (the active isometric state has not yet been investigated with $P_\perp$ vs wavelength for 1,5-IAEDANS at SH1). The probes differ in their inferences concerning the rigor and MgADP states. While IATR distinguishes rigor from MgADP, the IANBD at SH2 does not. This difference could result from the orientation of the probes in the cross-bridge such that the IANBD is oriented in such a way that it cannot sense the cross-bridge rotation due to the binding of MgADP. We think this is unlikely because the excitation spectra of IANBD for rigor and MgADP fibers are very similar throughout the wavelength scan. As pointed out above, this close similarity between rigor and MgADP throughout the spectrum requires the orientation of the probes to be aligned at all times during the gradual rotation of the transition dipole in the molecular frame as the wavelength varies.

The alternative explanation is simply that SH2 does not rotate upon the binding of MgADP as SH1 does. This explanation implies that the cross-bridge is not a rigid body. The idea of a nonrigid cross-bridge was used for a time to explain difference in the findings of probes on SH1. Although these particular differences are now resolved (Ajtai & Burghardt, 1987), the new data from SH2 again raise the possibility of flexibility within the cross-bridge. The idea of flexibility between SH1 and SH2 is not difficult to accept given that the transduction of energy between the ATP binding site and the actin binding site is thought to involve the displacement (not necessarily involving a rotation) of SH1 relative to SH2 as suggested by Förster energy-transfer experiments (Dalbey et al., 1983). Our data do not rule out the possibility that the presence of the IANBD on SH2 on the cross-bridge simulates a permanent ADP-like state in the cross-bridge (only so far as orientation is concerned) while not inhibiting the ATPases or force production.

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Registry No. MgADP, 7384-99-8; ATPase, 9000-83-3; IANBD, 67013-48-3.

REFERENCES

Ajtai, K., & Burghardt, T. P. (1986) *Biochemistry 25*, 6203–6207.
Ajtai, K., & Burghardt, T. P. (1987) *Biochemistry 26*, 4517–4523.
Bâlint, M., Srêter, F., Wolf, I., Nagy, B., & Gergely, J. (1975) *J. Biol. Chem. 250*, 6177-6188.
Bâlint, M., Wolf, I., Tarcsafalvi, A., Gergely, J. & Srêter, F. (1978) *Arch. Biochem. Biophys. 190*, 793–799.
Bârâny, M., Conover, T. E., Schliesefeld, L. H., Gaetjens, E., & Goffart, M. (1967) *Eur. J. Biochem. 2*, 156–164.
Borejdo, J., & Putnam, S. (1977) *Biochim. Biophys. Acta 459*, 578–595.
Borejdo, J., Putnam, S., & Morales, M. F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6346–6350.
Borejdo, J., Assulin, O., Ando, T., & Putnam, S. (1982) *J. Mol. Biol. 158*, 391–414.
Burghardt, T. P. (1984) *Biopolymers 23*, 2383–2406.
Burghardt, T. P., & Ajtai, K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8478–8482.
Burghardt, T. P., & Thompson, N. L. (1985) *Biophys. J. 48*, 401–409.
Burghardt, T. P., Ando, T., & Borejdo, J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7515–7519.
Crowder, M. S., & Cooke, R. (1984) *J. Muscle Res. Cell Motil. 5*, 131–146.
Crowder, M. S., & Cooke, R. (1987) *Biophys. J. 51*, 323–333.
Dalbey, R. E., Weiel, J., & Yount, R. G. (1983) *Biochemistry 22*, 4696–4706.
Duke, J., Takashi, R., Ue, K., & Morales, M. F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 302–306.
Green, L. (1986) *J. Biol. Chem. 26*, 1279–1285.
Huxley, A. F., & Simmons, R. M. (1971) *Nature (London) 233*, 533–538.
Huxley, H. E. (1969) *Science 164*, 1356–1366.
Huxley, H. E., & Kress, M. (1985) *J. Muscle Res. Cell Motil. 6*, 153–161.
Kameyama, T., Katori, T., & Sekine, T. (1977) *J. Biochem. 81*, 709–714.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Miki, M., & Wahl, P. (1984) *Biochim. Biophys. Acta 970*, 275–283.
Nihei, T., Menselson, R. A., & Botts, J. (1974) *Biophys. J. 14*, 236–242.
Reisler, E., Burke, M., & Harrington, W. F. (1974) *Biochemistry 13*, 2014–2022.
Rosenfeld, S. S., & Taylor, E. W. (1985) *J. Biol. Chem. 260*, 252–261.
Sekine, T., & Yamaguchi, M. (1963) *J. Biochem. 54*, 196–198.
Sekine, T., & Kielley, W. W. (1964) *Biochim. Biophys. Acta 81*, 336–345.
Sutoh, K. (1981) *Biochemistry 20*, 3281–3285.
Takashi, R., Duke, J., Ue, K., & Morales, M. F. (1976) *Arch. Biochem. Biophys. 175*, 279–283.
Thomas, D. D., & Cooke, R. (1980) *Biophys. J. 32*, 891–906.
Trybus, K. M., & Taylor, E. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7209–7213.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.
Yamaguchi, M., & Sekine, T. (1966) *J. Biochem. 59*, 24–33.
Yanagida, T. (1981) *J. Mol. Biol. 146*, 539–549.
Yanagida, T. (1985) *J. Muscle Res. Cell Motil. 6*, 43–52.

Phosphorus Nuclear Magnetic Resonance Studies of Lipid–Protein Interactions: Human Erythrocyte Glycophorin and Phospholipids†

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ABSTRACT: Human erythrocyte glycophorin containing four molecules of phospholipid tightly bound to the protein was isolated from human red cell ghosts. This protein preparation was reconstituted into a digalactosyl diglyceride bilayer. The ^{31}P NMR spectrum of this reconstituted membrane produced an axially symmetric powder pattern arising exclusively from the phospholipids bound to glycophorin. The width of the powder pattern, about 90 ppm, is about twice as broad as that normally exhibited by a phospholipid bilayer. The chemical shift tensor is perturbed relative to phospholipids in a bilayer. The spin–lattice relaxation rate of these protein-bound phospholipids is found to be nearly an order of magnitude faster than phospholipids in a bilayer. The results are consistent with phospholipids tightly bound to the membrane protein and undergoing rotational diffusion, perhaps as a complex of phospholipid and protein.

Lipid–protein interactions in membranes have been the subject of intense interest for a number of years because interactions between these two major components of biological membranes have been expected to be important to membrane function. However, before an adequate understanding of the potential roles of such interactions in cell membrane function can be obtained, a clear picture of the possible modes of interaction between lipids and proteins must be drawn.

It is reasonably well agreed that lipids and proteins in membranes must have some influence upon each other. For example, lipids (or their detergent replacements) are often required to activate membrane enzymes (Mitchell et al., 1983).

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