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# Differential Behavior of Two Cysteine Residues on the Myosin Head in Muscle Fibers†

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ABSTRACT: We have previously shown that the orientation of (iodoacetamido)tetramethylrhodamine labels on $SH_1$ thiol of S-1 moieties changes when MgADP is added to the fibers in rigor [Borejdo, J., Assulin, O., Ando, T., & Putnam, S. (1982) *J. Mol. Biol. 158*, 391–414. Burghardt, T. P., Ando, T., & Borejdo, J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7515–7519]. Here we report the results of experiments in which the $SH_2$ of S-1 was labeled with maleimidorhodamine. The specificity of modification of thiols was checked by measuring the stoichiometry of attached dye, by determining the extent of the decrease in EDTA($K^+$)- and $Ca^{2+}$-ATPase activities, and by the localization of the dyes on peptides containing $SH_1$ and/or $SH_2$. Labeled S-1 was diffused into single glycerinated fibers of rabbit psoas muscle, and the orientation of chromophores was measured by fluorescence detected dichroism. The dye attached to $SH_1$ was oriented at 65° with respect to the fiber axis in rigor and at 51° in the presence of MgADP, regardless of whether $SH_2$ was modified or not. The dye on $SH_2$ was oriented near 42° both in the presence and in the absence of ADP, regardless of whether $SH_1$ was modified or not. Our results show that rhodamine oriented differently when attached to $SH_2$ compared with when attached to $SH_1$ and that in the former placement it was not sensitive to MgADP. We think this indicates that the $SH_2$-containing region has a mobility different from that of the $SH_1$-containing region, i.e., that this is evidence for internal flexibility of S-1.

The central problem in muscle contractility is the question of how myosin heads (S-1[1] moieties of myosin) interact with actin. One approach in investigating this problem has been to measure the orientation of fluorescent or spin-labeled molecules attached to a reactive cysteine residue on S-1 of myosin and to follow the S-1 orientation at different physiological states [for reviews see Morales et al. (1982) and Cooke (1986)]. By use of the fluorescent dipoles of (iodoacetamido)tetramethylrhodamine (IAR) (Borejdo et al., 1982; Burghardt et al., 1983) or of *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (1,5-IAEDANS) (Ajtai & Burghardt, 1987) on the $SH_1$ residue of a myosin head to detect the polar angle of the absorbance dipole, it was found that the angle in the rigor state is different from that in the presence of nucleotides. The same change in angle also occurs when the temperature is reduced from >2 to <−10 °C (Ajtai & Burghardt, 1986). On the other hand, Thomas and Cooke (1980) reported that the EPR signal of a spin label [maleimido spin label (MSL)] on $SH_1$ is insensitive to MgADP. This raises the possibility that some probes (e.g., IAR, 1,5-IAEDANS) rotate through a favorable angle for detecting angular motion, while others (e.g., MSL) rotate through an unfavorable angle. Alternatively, the ring system of one probe could rest on a structural niche (or domain) that moves, while the ring system of another could rest on a niche (or domain) that does not move.

In this paper, we modified $SH_1$ and $SH_2$ with several reagents in an attempt to compare the angular reorientations of $SH_1$- or $SH_2$-labeled regions of S-1 in response to the addition of nucleotides. In both cases, modified S-1 retained actin- and ATP-binding abilities. IAR was located almost exclusively on the $SH_1$ residue and maleimidotetramethylrhodamine (MLR) on the $SH_2$ residue of S-1. Labeled S-1 was diffused into single glycerinated rabbit psoas fibers in rigor solution and bound to the I-bands. Following addition of MgADP there was a change in dipole orientation for a dye attached to $SH_1$ but not for a dye on $SH_2$. The dipoles were found to be firmly attached to the protein, and hence we

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[1] Abbreviations: S-1, myosin subfragment 1; $SH_1$, reactive cysteine residue 1; $SH_2$, reactive cysteine residue 2; FDNB, 2,4-dinitrofluorobenzene; IAA, iodoacetamide; NEM, *N*-ethylmaleimide; IAR, (iodoacetamido)tetramethylrhodamine; 1,5-IAEDANS, *N*-[[(iodoacetyl)-amino]ethyl]-5-naphthylamine-1-sulfonic acid; MSL, maleimido spin label; MLR, maleimidotetramethylrhodamine; ϵ-ATP, 1,$N^6$-ethenoadenosine triphosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; kDa, kilodaltons; LD, linear dichroism; FP, fluorescence polarization = polarization of fluorescence.

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conclude that the intramolecular movement caused by nucleotides "around $SH_1$" is different from that around $SH_2$, suggesting that S-1 is internally flexible.

## Experimental Procedures

*Proteins, Modification, Muscle Fibers, and ATPase Assay.* Myosin was prepared from rabbit skeletal muscle by the method of Tonomura et al. (1966). S-1 was obtained by chymotryptic (Weeds & Taylor, 1975) or papain digestion (Margossian & Lowey, 1982) of myosin filaments. Actin was prepared according to the method of Spudich and Watt (1971).

$SH_1$ was modified by incubation of S-1 or myosin with a 1.5-fold excess of IAR for 12 h or with a 4-fold excess of iodoacetamide (IAA) for 12 h at 0 °C. Alternatively $SH_1$ was modified by addition of fluorodinitrobenzene (FDNB) to the protein in 1 mM Mg-ADP, 0.5 M KCl, and 50 mM Tris-HCl at pH 7.8 for 20 min at 0 °C (Reisler, 1982). In each case, excess reagents were removed by washing myosin with 10 mM TES, pH 7.0, or by dialysis. $SH_2$ was modifed by incubation of $SH_1$-modified S-1 or of myosin with a 2-fold excess of MLR or a 4-fold excess of NEM in 1 mM Mg-ADP, 0.5 KCl, and 50 mM Tris-HCl at pH 7.8 and 0 °C for 12 h. DNP was removed from S-1 by 10 mM DTT in 0.5 M KCl and 20 mM Tris-HCl at pH 8.0 and 25 °C for 3–12 h.

In order to check whether the fluorescent label was located on $SH_1$ or $SH_2$ of S-1, modified S-1 was subjected to trypsin cleavage in 1% w/w trypsin, 0.1 M NaCl, 20 mM TES, and 1 mM EDTA at pH 7.0 and 20 °C for 2–15 min, to $NH_2OH$ cleavage in 1 M $NH_2OH$ and 6 M Gdn-HCl at pH 9.0 with LiOH and 40 °C for 1–8 h (Bornstein & Balian, 1977), or to CNBr cleavage in 70% formic acid containing a 500-fold excess of CNBr for 12 h at 25 °C. The products were run on SDS–PAGE (Laemmli, 1970), and the quantity of fluorescent label associated with each fragment was estimated.

ATPase activities were measured in 0.5 M KCl, 50 mM Tris-HCl, 5 mM EDTA, or 5 mM $CaCl_2$ at pH 7.8 and 20 °C. The resultant $P_i$ concentrations were measured by using a modified Fiske and Subbarow (1925) method. Binding of ADP to actin-modified S-1 complex was examined by using a fluorescent $\epsilon$-ADP. $\epsilon$-ATP is known to be hydrolyzed by myosin, leading to a formation of M·ADP·P complex, and to support muscle contraction (Onishi et al., 1973; Mowery, 1973). $\epsilon$-ADP (40–80 $\mu$M) was incubated with 20–40 $\mu$M S-1 and 30–60 $\mu$M actin in 0.1 M NaCl, 20 mM TES, and 2 mM $MgCl_2$ at pH 7.0 and 20 °C. After ultracentrifugation at 250000*g* for 30 min (almost $\epsilon$-ADP S-1 was precipitated with actin as checked by SDS–PAGE), the amount of free $\epsilon$-ADP in the supernatant was calculated from its fluorescent intensity. Under the conditions used, the fluorescent intensity of $\epsilon$-ADP was proportional to its concentration. Knowing the amount of $\epsilon$-ADP added, we calculated the amount of $\epsilon$-ADP bound to acto-S-1 complex. The results were corrected for the fluorescence of residual S-1 and actin in the supernatant. This amount was obtained by the control ultracentrifugation of acto-S-1 with and without MgADP.

Single glycerinated muscle fibers from rabbit psoas muscle were used and mounted as described before (Borejdo et al., 1982). Modified S-1 was added to whole fibers without extracting myosin in order to preserve the high regular arrangement of thin filaments, which could be disrupted by high ionic strength myosin-extracting solution.

Bound label was estimated both from the change in ATPase activity that it produced and from absorbance measurements. Bound label on $SH_1$ was estimated by the decrease in EDTA($K^+$)-ATPase activity, and bound label on $SH_2$ of $SH_1$-modified S-1 was estimated from the observed decrease in $Ca^{2+}$-ATPase activity of $SH_1$-modified S-1. IAR has different absorption coefficients ($\epsilon$) (and dichroic spectra, see below) depending on the source. For IAR from Molecular Probes (Eugene, OR) (Takashi & Kasprzak, 1987) $\epsilon^{555}$ was $50 \times 10^3$ $M^{-1}$ $cm^{-1}$; for IAR from Research Organics (Cleveland, OH) (Tait & Frieden, 1982) $\epsilon^{554}$ was $23 \times 10^3$ $M^{-1}$ $cm^{-1}$. It should be noted that Takashi and Kasprzak (1987) have determined the extinction coefficient of IAR from Molecular Probes and showed that the IAR labeling is specific to $SH_1$. $\epsilon^{360}$ for the dinitrophenyl (DNP) group was taken as $14.8 \times 10^3$ $M^{-1}$ $cm^{-1}$.

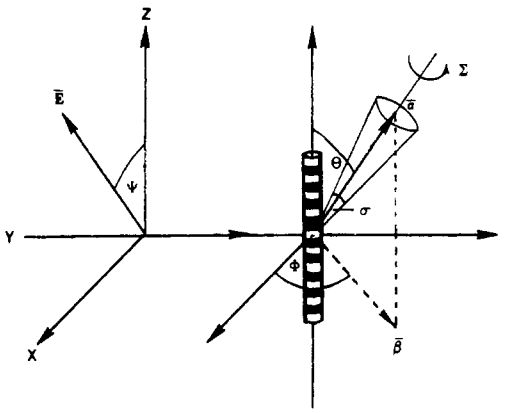


FIGURE 1: Diagram illustrating the geometry of the dichroism experiment. **E** is the unit vector in the direction of polarization of exciting light, forming angle $\psi$ with the vertical in the $Y$–$Z$ plane. $\alpha$ is the absorption dipole of the chromophore forming an inclination angle $\theta$ with the $Z$ axis. Projection of this vector on the $X$–$Y$ plane is $\beta$, and the angle between $\beta$ and the $X$ axis is an azimuthal angle $\phi$. $\alpha$ can rotate by (torsional) angle, $\Sigma$, along its axis. $\sigma$ is an angle defining a cone within which $\alpha$ can rotate freely.

*Actin Binding.* Binding of actin to modified S-1 was monitored by light scattering at 90° from incident light, using wavelengths of 340 or 400 nm in a Perkin-Elmer MPF-4 fluorescence spectrophotometer. The solution contained 4 $\mu$M actin, 0–9 $\mu$M S-1, 0.1 M KCl, and 20 mM TES, 2 mM $MgCl_2$ at pH 7.0 and 25 °C.

*Dichroic Measurements.* A single glycerinated muscle fiber from rabbit psoas muscle (Borejdo et al., 1979) was placed on a slide and positioned horizontally on the stage of a light microscope (Zeiss Photomicroscope III). The fiber was bathed in 0.1 M sodium acetate, 5 mM $MgCl_2$, 10 mM EGTA, and 20 mM TES at pH 7.0 and 25 °C. The fiber was then incubated in a bathing medium containing modified S-1 (30–50 $\mu$M) for 15–30 min. Excess S-1 was washed out by replacing the bathing medium for the one without S-1. The dye was excited by light from an argon ion laser (Spectra Physics Model 164-03) at a wavelength of 514.5 nm unless otherwise stated. Figure 1 defines the geometry of the experiment. The exciting light **E** at $\psi$ angle irradiates the absorption dipole $\alpha$ oriented at an average angle of $\theta$ with a deviation angle $\sigma$, i.e., the deviation of the absorption dipole is assumed to be distributed with equal probability around the mean angle $\theta$. The resultant fluorescence from the fiber was detected as described by Borejdo et al. (1982). The plane of polarization of exciting light (the angle $\psi$ in Figure 1) was rotated between directions parallel and perpendicular to the fiber axis by a Pockels cell (Lasermetrics Model 3079FW) with a single end configured amplifier (Lasermetrics, GA-21) controlled by a ramp generator (Exact Model 126). To compensate for polarization of emitted light by the dichroic mirror, which was at an angle of 45° to the direction of propagation of light (Jenkins & White, 1957), a second dichroic mirror was inserted above the first one, which was also inclined at 45° with respect to the

Table I: Dyes Bound to Thiols of S-1 and Their Effects on ATPase Activities

| reagents[a] | | ATPase activities[b] ($min^{-1}$) | | calcd extent of modification (mol/mol) | | amount of dye found[d] (mol/mol) | |
|---|---|---|---|---|---|---|---|
| $SH_1$ | $SH_2$ | EDTA ($K^+$) | $Ca^{2+}$ | $SH_1$ | $SH_2$ | $SH_1$ | $SH_2$ |
| – | – | 320 | 92.8 | 0 | 0 | 0 | 0 |
| DNP | – | 45.6 | 230 | 0.86 | – | 0.89 | ND[e] |
| DNP | MLR* | 12.9 | 88.9 | | 0.62 | 0.89 | 0.7 |
| IAR* | – | 174 | 152 | 0.46 | – | 0.51 | ND[e] |
| IAR* | NEM | 4.6 | 19.0 | | 0.88 | 0.51 | ND[e] |
| MLR* | – | 258 | 96.2 | 0.19 | – | 0.63 | ND[e] |
| DNP/DTT[f] | – | 277 | 94.6 | 0.13 | – | 0.05 | ND[e] |
| DNP/DTT | MLR* | 216 | 70.0 | 0.32 | ND[e] | 0.1 | 0.7 |

[a] Modification of $SH_1$ and/or $SH_2$ was performed as described under Experimental Procedures with dinitrofluorobenzene (DNP), maleimidotetramethylrhodamine (MLR), (iodoacetamido)tetramethylrhodamine [IAR, previously referred to as IATR by Borejdo et al. (1982)], or *N*-ethylmaleimide (NEM). The asterisk indicates that the substance is fluorescent. A dash indicates that the measurement is not applicable. [b] EDTA ($K^+$)- and $Ca^{2+}$-ATPase activities were measured in 1 $\mu$M S-1, 2 mM ATP, 0.5 M KCl, and 50 mM Tris-HCl at pH 7.8 and 25 °C, containing 5 mM EDTA or 5 mM $CaCl_2$, respectively. [c] The extent of $SH_1$ modification was estimated from the decrease of EDTA ($K^+$)-ATPase activity and that for $SH_2$ from the decrease of $Ca^{2+}$-ATPase activation induced by $SH_1$ label Reisler et al., 1982). [d] Bound DNP was determined by assuming $\epsilon^{360} = 14.8 \times 10^3\ M^{-1}\ cm^{-1}$. Bound MLR or IAR was determined by assuming $\epsilon^{555} = 50.0 \times 10^3\ M^{-1}\ cm^{-1}$. [e] ND indicates that the quantity was not determined. [f] DNP/DTT denotes removal of DNP from $SH_1$ by DTT.

direction of propagation of light but rotated by 90° with respect to the first mirror in a plane perpendicular to the direction of propagation of light.

*Data Analysis.* Equation 1 (Borejdo & Burghardt, 1987) relates the absorption (**A**) and the chromophore dipole angle $\theta$ and its deviation $\sigma$:

$$\mathbf{A} = ax_0(\psi) + cx_2(\psi) \quad (1)$$

$$x_0 = \cos^2 \psi - \tfrac{1}{2} \sin^2 \psi$$

$$x_2 = -\tfrac{1}{2} \sin^2 \psi$$

$$a = \tfrac{2}{9} + \tfrac{2}{9}[\sin^2 (\theta + \sigma) \cos (\theta + \sigma) - \sin^2 (\theta - \sigma) \cos (\theta - \sigma)/\cos (\theta - \sigma) - \cos (\theta + \sigma)]$$

$$c = -\tfrac{2}{3}$$

To determine the average polar angle $\theta$, the fiber absorption ($A$) at different orientations of exciting light ($\psi$), and a cone angle $\sigma$ have to be determined. The extent of absorption $A$ at each $\psi$ was obtained from the amount of fluorescence. The cone angle $\sigma$ is the angle of free rotation of the dipole of the dye on the surface of S-1 and can be calculated from the decrease in time zero polarization of fluorescence. If there were no local, depolarizing motion of the dye and if the protein segment to which the probe is attached were rigid, the polarization for the S-1 should be equal to $p_0$ because one can neglect the motion of the F-actin ($M_r > 10^6$). As can be seen from Table III, this is not the case and the limiting polarization of S-1 attached to F-actin (in the absence of sucrose) has lower value, indicating that a rapid rotation of the chromophore takes place. The angle of the rotation $\sigma$ is related to the ratio of limiting polarization in the absence of sucrose ($p_x$) to time zero polarization ($p_0$) by (Weber, 1966)

$$p_x/p_0 = (3 \cos^2 \sigma - 1)/2 \quad (2)$$

$\phi$ and $\Sigma$ in Figure 1 are respectively the azimuth angle, i.e., the projection of the absorption vector $\alpha$ into $X$–$Y$ plane, and the torsional angle, i.e., the angle of rotation of vector $\alpha$ around it own axis. $\phi$ does not enter eq 1 because the spherical symmetry of the arrangement of the dipoles with respect to the fiber axis eliminates dependence of **A** on the azimuthal angle (Borejdo et al., 1982). Since torsional movement of the dipole is not considered in this model, the angle $\Sigma$ does not appear in eq 1. Angles $\phi$ and $\Sigma$ are treated under Discussion.

In experiment, **A** is measured as a function of $\psi$. $\sigma$ is calculated from eq 2, and $\theta$ is calculated by using the ratio of **A** at $\psi = 90°$ to **A** at $\psi = 0°$ from eq 1.

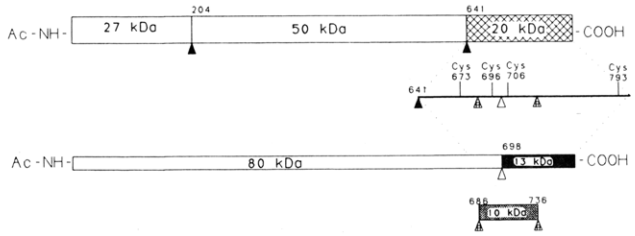


FIGURE 2: Schematic presentation of location of $SH_1$ and $SH_2$ in the amino acid sequence of chymotryptic S-1 heavy chain. $SH_1$ and $SH_2$ are Cys 706 and Cys 696, respectively. The sequence number of S-1 heavy chain on the lines is referred to Maita et al. (1987). Cleavage sites by trypsin, hydroxylamine, or cyanogen bromide cleavage, are shown as solid, open, or hatched triangles, respectively, under the sequence. The size of each resultant fragment is given in each box of the sequence. C-Terminal tryptic 20-kDa and hydroxylamine 13-kDa fragments will be 26- and 19-kDa fragments, respectively, when we use papain S-1.

## Results

It is well established that the extent of $SH_1$ modification is nearly proportional to the decrease in EDTA($K^+$)-ATPase activity and that the extent of $SH_2$ modification is approximately proportional to the decrease of the $Ca^{2+}$-ATPase activity enhanced by $SH_1$ modification (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966). One can therefore estimate the extent of SH group modification by the changes in these ATPase activities. Table I summarizes the effect of thiol modification on ATPase activities and compares the extent of modification estimated by ATPase changes with that estimated from absorbance of bound dyes. In Table I, the amount of dye found was assigned to $SH_1$ when the conditions giving $SH_1$ modification were used and to $SH_2$ when the conditions giving $SH_2$ modification were used. With the exception of lines 6 and 8, there is good agreement between these two estimates, supporting the idea that $SH_1$ and $SH_2$ were modified specifically under the conditions used. The poor agreement between the two estimates of bound label observed for MLR labeling of $SH_1$ (line 6) shows an ambiguity about the specificity of MLR for selective modification of $SH_1$. The difference for the two estimates when $SH_1$ was modified with DNP and $SH_2$ with MLR (line 3), followed by DNP removal from the $SH_1$ by DTT (line 8), suggests that after labeling $SH_2$, the EDTA($K^+$)-ATPase activity cannot be completely recovered. This is consistent with the results obtained by Reisler and Burke (1974).

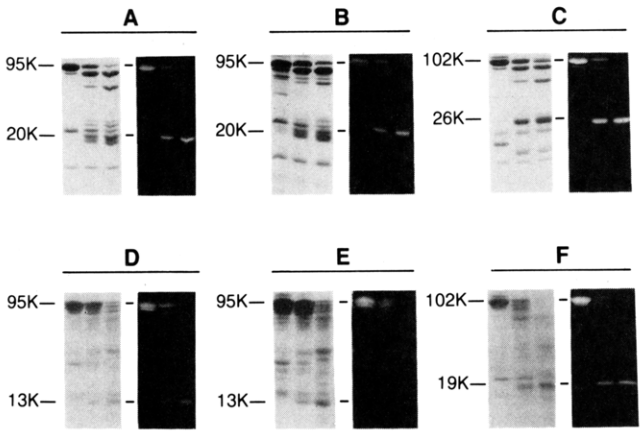


FIGURE 3: Location of fluorescent dye on $SH_1$ and $SH_2$ residues in heavy chain of S-1. Limited trypsin digestion (A–C) or hydroxylamine cleavage (D–F) of modified S-1 was followed by SDS–PAGE. A pair of photographs is given for each sample; one for Coomassie staining, the other for fluorescent detection. Each pair of photographs gives images of 0-, 2-, and 4-min trypsin digestion of S-1 in (A)–(C) or 0-, 1-, and 2-hydroxylamine cleavage in (D)–(F). (A and D) Chymotryptic S-1 with IAR on $SH_1$; (B and E) chymotryptic S-1 with MLR on $SH_2$; (C and F) papain S-1 with IAR on $SH_1$.

These specificities need to be confirmed by location of the dye in peptides of the heavy chain. Including $SH_1$ and $SH_2$, there are four cysteine residues in the 20-kDa part of the S-1 heavy chain as shown in Figure 2. $SH_1$ and $SH_2$ are found separately from the other residues in the 10-kDa CNBr fragment, and cleavage of S-1 with $NH_2OH$ produces a 13-kDa fragment containing $SH_1$ and a second larger fragment with $SH_2$. $SH_1$ and $SH_2$ were modified as described under Experimental Procedures. Figure 3 shows the time course of degrading the S-1 heavy chain by trypsin and by $NH_2OH$, as revealed by SDS–PAGE. When S-1 was modified under conditions in which one expects $SH_1$ to be labeled, the fluorescent label was mostly found on the tryptic 20-kDa and on the $NH_2OH$ 13-kDa fragments (Figure 3A,C,D,F). In the case of $SH_2$ modification, the label was again found on the 20-kDa tryptic fragment. However, it was no longer on the 13-kDa $NH_2OH$ fragment but instead on the larger $NH_2OH$ fragments of S-1 and their degradation products (which are distinguishable from the 100-kDa fragment, Figure 3B,E) (Sutoh, 1981). Upon CNBr cleavage for both S-1 modifications, fluorescence was observed for the 10-kDa segment, showing that only thiols which had been modified were either $SH_1$ or $SH_2$ (see Figure 2). These results suggest that we can selectively modify $SH_1$ or $SH_2$ with these fluorescent dyes. Similarly, the specificity of modification of $SH_2$ by MLR in S-1 in which IAA was used to block $SH_1$ and the specificity of modification of $SH_1$ by IAR in S-1 in which NEM was used to block $SH_2$ were confirmed by peptide mapping. Modification of $SH_2$ with IAR resulted in a significant modification of light chains as checked by SDS–PAGE.

The actin-binding affinity of S-1 was approximately $1 \times 10^7$ $M^{-1}$ when measured by the light-scattering method under the conditions used for orientation measurements, i.e., 0.1 M NaCl, 20 mM TES, and 5 mM $MgCl_2$ (Figure 4). In agreement with earlier papers (Highsmith et al., 1976; Botts et al., 1979) modification of $SH_1$ resulted in a slight loss of actin-binding affinity. Further modification of $SH_2$ did not cause any large alterations in this affinity (Figure 4). Modification of $SH_1$ with DNP and with MLR gave similar results (Figure 4). Addition of excess MgATP to those complexes reversed the increase in scattering associated with binding to actin, suggesting that modified S-1 used here retained its

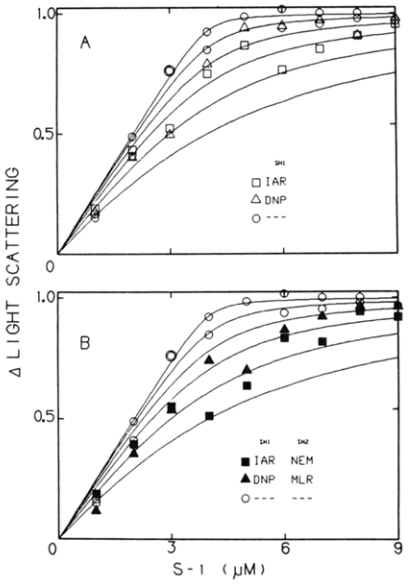


FIGURE 4: Actin binding to S-1, using native, $SH_1$- and/or $SH_2$-modified S-1, monitored by the light-scattering method: 4 $\mu$M actin, 0–9 $\mu$M S-1 in 0.1 M sodium acetate, 20 mM TES, 4 mM $MgCl_2$ at pH 7.0 and 25 °C. The light-scattering intensity of the acto-S-1 mixture was detected at 340 nm; from this intensity was subtracted the sum of scattered intensity of S-1 alone and actin alone obtained separately, which were not dependent on the modification of thiols. The maximum binding is assumed to be given when more than 9 $\mu$M of native S-1 was added to 4 $\mu$M actin, since the binding constant of acto-S-1 at the present condition has been already reported to be $10^7$ $M^{-1}$ (Marston & Weber, 1975; White & Taylor, 1976). The continuous curves were computer simulated by using $1/K_a = ([A_t] - [AM])([M_t] - [AM])/[AM]$, while $K_a$ (from top to bottom) is $4 \times 10^7$, $1 \times 10^7$, $4 \times 10^6$, $2 \times 10^6$, $1 \times 10^6$, and $5 \times 10^5$ $M^{-1}$, where [AM], $[A_t]$, and $[M_t]$ are concentrations of acto-S-1, total actin (4 $\mu$M), and total S-1 (0–9 $\mu$M), respectively. In fact, the theoretical curve for $K_a = 10^7$ $M^{-1}$ fits well the experimental value for unmodified S-1. (A) ○, unmodified S-1; △, S-1 with DNP on $SH_1$; □, S-1 with IAR on $SH_1$. (B) ○, unmodified S-1; ▲, S-1 with DNP on $SH_1$ and with MLR on $SH_2$; ■, S-1 with IAR on $SH_1$ and with NEM on $SH_2$.

ATP-binding ability. The binding of MgADP to acto-S-1 complex was examined by using $\epsilon$-ADP as described under Experimental Procedures. When S-1 was modified at $SH_1$ with IAR, the affinity of $\epsilon$-ADP to acto-S-1 complex was found to be about $7 \times 10^3$ $M^{-1}$ from two measurements (Table II). When S-1 was modified at $SH_1$ with DNP and at $SH_2$ with MLR, the affinity of $\epsilon$-ADP to acto-S-1 complex was found to be about $6 \times 10^3$ $M^{-1}$ from two measurements (Table II). These values agree well with the association constant of ADP to acto-S-1 complex or to myosin head in myofibrils measured by Green and Eisenberg (1980), by Highsmith (1976), and by Johnson and Adams (1984) and suggest that 1 mM MgADP used in our experiments is sufficient to bind to most of the modified S-1 in the muscle fiber.

In determinations of $\theta$, polarization of fluorescence at time zero ($p_0$) was measured first in order to compute $\sigma$ by using eq 2. To this end, polarization of fluorescence $p$ was measured in the presence of various concentrations of sucrose (10–50%, w/v), and the result was plotted as Perrin plots (Weber, 1952); i.e., $1/p$ was plotted as a function of $1/\eta$, where $\eta$ is the solvent viscosity. The extrapolation of $p$ to infinite viscosity yields $p_0$ or $\rho$, the molecular rotational relaxation time (Pesce et al., 1971). $p_x$ was taken as a value of $p$ in the absence of added sucrose but in the presence of F-actin (to immobilize S-1). Table III summarizes the results obtained for IAR probe placed on $SH_1$ and MLR probe on $SH_2$ of S-1. It can be seen that they both were immobilized within fairly small cone angles on the surface of S-1 (23° for IAR on $SH_1$ and 21° for MLR

Table II: ϵ-ADP Binding to Acto-S-1 Complex

| labels | | rel fluorescence in the supernatant after ultracentrifugation[a] | | | |
|---|---|---|---|---|---|
| $SH_1$ | $SH_2$ | ϵ-ADP | S-1 + actin | S-1 + actin + ADP | S-1 + actin + ϵ-ADP |
| IAR[b] | | 154 | 43 | 44 | 182 |
| IAR[c] | | 309 | 85 | 84 | 344 |
| DNP[b] | MLR | 155 | 28 | 29 | 170 |
| DNP[c] | MLR | 308 | 50 | 50 | 315 |

| labels | | calcd concn (μM) | | | |
|---|---|---|---|---|---|
| $SH_1$ | $SH_2$ | free ϵ-ADP | bound ϵ-ADP | ϵ-ADP–free S-1 | calcd $10^3K_a$ ($M^{-1}$) |
| IAR[b] | | 36.1 | 3.9 | 16.1 | 6.7 |
| IAR[c] | | 67.3 | 12.7 | 27.3 | 6.9 |
| DNP[b] | MLR | 36.4 | 3.6 | 16.4 | 6.0 |
| DNP[c] | MLR | 68.8 | 11.2 | 28.8 | 5.7 |

[a] The solution of ϵ-ADP, S-1 + actin, S-1 + actin + ADP, or S-1 + actin + ϵ-ADP was incubated at pH 7.0 and 20 °C as described under Experimental Procedures and centrifuged at 250000*g* for 30 min. The fluorescence of the resulting supernatant was monitored at 274-nm excitation and 416-nm emission. Since the supernatant of S-1 + actin + ADP showed detectable fluorescence as did that of S-1 + actin + ADP (DNP on $SH_1$ seemed to absorb some fluorescence of S-1), the fluorescence of S-1 + actin + ϵ-ADP was subtracted from that of S-1 + actin + ADP to estimate the true fluorescent content of free ϵ-ADP in the S-1 + actin + ϵ-ADP mixture. The value was then divided by the fluorescence of the supernatant of ϵ-ADP alone and multiplied by the concentration of ϵ-ADP alone (40 or 80 μM) to give the values of free ϵ-ADP in the lower section of this table. ϵ-ADP was not detected to cosediment with actin alone (data not shown). Then bound ϵ-ADP was given by subtraction of the concentration of total ϵ-ADP from that of free ϵ-ADP. ϵ-ADP–free S-1 was calculated by subtraction of S-1 in the pellet (almost equal to that of total S-1) from bound ϵ-ADP. $K_a$ is [bound ϵ-ADP]/([free ϵ-ADP][ϵ-ADP–free S-1]). [b] 20 μM S-1 with IAR on $SH_1$ or with DNP on $SH_1$ and MLR on $SH_2$ as obtained in Table I was incubated with 30 μM actin and 40 μM ϵ-ADP and ultracentrifuged, and the fluorescence ind the resulting supernatant was monitored as described under Experimental Procedures and the above. [c] The procedures were the same as in the above experiment except that 40 μM S-1 with labels was incubated with 60 μM actin and 80 μM ϵ-ADP.

Table III: Summary of Static Polarization of Fluorescence of Dyes on Thiols of S-1[a]

| | $p_0$ | | $p_x$ | ρ (ns) | | σ (deg) | |
|---|---|---|---|---|---|---|---|
| | rigor[b] | ADP[c] | rigor | rigor | ADP | rigor | ADP |
| IAR ($SH_1$) | 0.400 | 0.400 | 0.310 | 150 | 140 | 23 | 23 |
| MLR ($SH_2$) | 0.364[d] | 0.364[d] | 0.292 | 43 | 42 | 21 | 21 |

[a] Polarization of modified S-1 (4 μM) was monitored in 0.1 M sodium acetate, 20 mM TES, and 2 mM $MgCl_2$ at pH 7.0 and 20 °C containing 0–50% (w/v) sucrose and was calculated by using eq 2. [b] Here "rigor" is used to indicate "no nucleotide added", which is the same conditions as used for the dichroic measurement. [c] MgADP was added at 1 mM to the medium. [d] We note that since the decrease in $p_0$ from the maximum value of 0.5 is due only to the misalignment of absorption and emission dipoles, its value should not depend on a position of the dye on the molecule. In other words, ideally it should be the same regardless of whether the dye is on $SH_1$ or $SH_2$. The fact that we repeatedly observed the difference in $p_0$ for S-1 labeled on $SH_1$ and $SH_2$ suggests that labeling distorts the structure of the dye.

on $SH_2$). The relaxation time was estimated to be 140–150 ns for IAR on $SH_1$ and 42–43 ns for MLR on $SH_2$, suggesting that IAR oriented along the longer axis of S-1 while MLR oriented mainly along its shorter axis.

Figure 5 shows the dependence of fluorescence of a muscle fiber irrigated with IAR $SH_1$-labeled S-1 on the angle ψ between the muscle axis and the direction of polarization of laser light. As shown previously (Borejdo et al., 1982), when the fluorescence is detected with a high numerical aperture objective lens as was the case here, the observed fluorescence is proportional to total fluorescence emitted by the fiber and

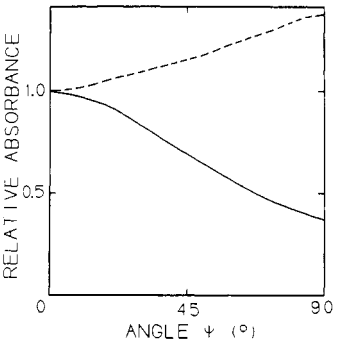


FIGURE 5: Typical curve for dichroic measurement of S-1 with fluorescent dye on $SH_1$ in a muscle fiber. Dichroism was monitored by varying the angle of the polarized exciting beam from 0° to 90° with respect to the *Z* line of the fiber. (—) Measured at rigor state; (– – –) measured at MgADP state.

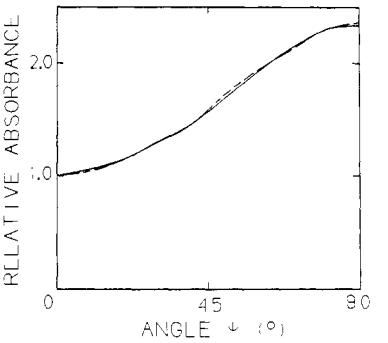


FIGURE 6: Typical curve for dichroic measurement of S-1 with fluorescent dye on $SH_2$. (—) At rigor state; (– – –) at MgADP state.

Table IV: Orientation of Dyes Attached to Thiols of S-1

| site[a] | | angle[b] (deg) | | excitation wavelength (nm) |
|---|---|---|---|---|
| $SH_1$ | $SH_2$ | rigor | ADP | |
| IAR* | | 65.4 + 2.6[c] | 51.6 + 1.9 | 514.5 |
| IAR[d] | | 57.3 ± 1.1 | 50.5 ± 1.4 | 514.5 |
| IAR[e] | | 64.0 ± 3.2 | 47.3 ± 2.0 | 514.5 |
| MLR* | | 57.5 ± 2.5 | 47.7 ± 1.4 | 514.5 |
| IAR* | | 66.0 | 52.5 | 488.0 |
| IAR* | | 60.0 | 53.0 | 457.9 |
| IAR* | NEM | 66.0 ± 2.8 | 55.0 ± 1.4 | 514.5 |
| IAA | MLR* | 41.0 ± 2.1 | 39.9 ± 2.7 | 514.5 |
| DNP | MLR* | 41.8 ± 3.5 | 42.0 ± 3.4 | 514.5 |
| DNP/DTT | MLR* | 42.7 ± 2.8 | 39.8 ± 3.7 | 514.5 |
| DMP | MLR* | 44.0 | 43.0 | 488.0 |
| DNP | MLR* | 44.5 ± 0.7 | 45.0 ± 0.4 | 457.9 |

[a] Sequence locations of dyes were examined by SDS–PAGE of peptides as shown in Figure 1 except MLR on $SH_1$, which used a method of modification similar to that for IAR. The asterisk indicates fluorescent labels. [b] Angles were calculated to fit the data by eq 1. These angles are the averages of several reproducible experiments on different preparations of modified S-1. [c] Standard deviation of experiments done on two to eight different muscle fibers. [d] This experiment used papain S-1 instead of chymotryptic S-1. [e] IAR used here was purchased from a different source: Research Organics, Co.

hence is proportional to the absorbance. The average angle of dipole of the dye was at 65° to the filament axis in rigor and at 51° in the presence of ADP (Table IV). This compares well with the angles of 69° in rigor and 47° in the presence of ADP for $SH_1$-labeled myosin in muscle fibers (Burghardt et al., 1983). When $SH_1$ on S-1 was labeled with IAR obtained from Research Organics, the angles in rigor and in the presence of ADP were slightly different (Table IV). When the fiber was irrigated with $SH_2$-labeled S-1, angle θ was around 41° in both the presence and absence of ADP (Figure 6, Table IV), although the nucleotide-binding ability of modified S-1 was intact. Table IV summarizes orientations

of dye dipoles on $SH_1$ or $SH_2$ in various samples. In general, the orientation of the dye dipole on $SH_1$ was similar irrespective of whether $SH_2$ was modified, but changed upon addition of ADP. Orientation of the dye dipole on $SH_2$ was insensitive to ADP even with DNP removed from $SH_1$. It proved impossible to specifically label only $SH_2$ without first blocking $SH_1$. The absorption curve of MLR on $SH_2$ changed upon the addition of ATP or AMPPNP, giving the change angle $\theta$ from about 42° to about 50°. Similarly, the orientation of IAR on $SH_1$ changed from about 65° to about 56° upon the addition of ATP or AMPPNP. The addition of ATP or AMPPNP is known to dissociate myosin heads from actin filaments. These results indicate the possibility that ATP and AMPPNP partly dissociate S-1 from actin filaments and randomize the orientation of dipoles (i.e., they become closer to the "magic" angle of 54.7°, where we cannot distinguish the difference between random and ordered orientations).

## Discussion

The experimental approach we have used here, namely Pockels cell driven linear dichroism (LD) of muscle fibers irrigated with exogenous S-1, has two advantages over conventional measurements. (1) Labeling is specific to S-1, because S-1 is labeled in vitro by using techniques applicable only in solution (Takashi & Kasprzak, 1987; Morales et al., 1982; Reisler, 1982) (Figure 3). In particular, $SH_2$ can be specifically labeled, which is not easily achieved in muscle fibers. (2) The direction of polarization of the exciting light is rapidly varied by the Pockels cell, and the dichroic method allows light collection with a high efficiency as previously described (Borejdo et al., 1982). Therefore, the whole measurement can be completed in a fraction of a second. These factors combined to allow rapid measurements (for example, within 1 $\mu$s) with a high signal-to-noise ratio and with illuminating light intensity of less than $2 \times 10^{12}$ photons/s (<1 $\mu$W at 514.5 nm). In practice, we made dynamic and static measurements in 1 ms and 1 s, respectively. Specific labeling gives high orientation of chromophores, and low levels of illumination and short exposure time prevent photobleaching. Together these factors combined to give good agreement between the experimental results and theory.

Single muscle fibers irrigated with S-1 whose $SH_1$ was labeled with IAR gave $\theta = 65.4 \pm 2.6°$ in rigor and $\theta = 51.6 \pm 1.9°$ in the presence of MgADP (Table IV) for the orientation of the dye.

When the dye was attached to $SH_1$ via an iodoacetyl group and to $SH_2$ via a maleimide group, the angles of the dipoles on $SH_1$ and $SH_2$ were different (Table IV). Furthermore, MgADP influences eath thiol differently. In agreement with previous papers (Borejdo et al., 1982; Burghardt et al., 1983), the addition of MgADP to fibers irrigated with S-1 labeled with rhodamine dye on $SH_1$ caused a significant change in the dipole orientation of the dye without destroying the high degree of order present in rigor. In contrast, orientation of fluorescent dipoles on $SH_2$ was almost insensitive to the presence of MgADP (Figure 6). The simplest interpretation of this result is that the region of S-1 where $SH_1$ and $SH_2$ are located does not behave as a rigid body. If it was rigid, both thiols would have to experience similar change since they are close together: they are both located in the 20-kDa domain (Balint et al., 1978), and they are separated only by nine amino acid residues in the primary sequence (Elzinga & Collins, 1977; Maita et al., 1987). They are close enough to be cross-linked by DTNB in the presence of MgADP (Wells & Yount, 1980). The view that S-1 is internally flexible, i.e., that the $SH_1$-containing part rotates by the binding of MgADP while the $SH_2$-containing part is orientationally fixed with respect to the actin filament, is consistent with the fact that some fluorescent probes on $SH_1$ respond to MgADP but others in which the chromophoric group is placed on a different region on the surface of S-1 do not (Borejdo et al., 1982). Cooke (1986) interpreted EPR data in terms of flexibility of the cross-bridge during muscle contraction.

It is possible that there is movement of the $SH_2$ group and its vicinity but in a direction that is undetectable by the dichroic or polarization of fluorescence methods. For example, motion restricted to the change in the azimuthal angle ($\phi$, Figure 1) with respect to the fiber axis will not be detected by any probe method because of the cylindrical symmetry of the dipoles around the fiber axis. Similarly, if the change in the so-called torsional "twist" angle ($\Sigma$, Figure 1) (Morales et al., 1982) does not involve any change in the polar angle, this motion will also be undetected. It is also possible that there is no rotational motion in the vicinity of $SH_2$ but only translational motion that does not affect the polar angle ($\theta$, Figure 1). However, for two reasons, we believe that none of these possibilities are likely, i.e., we believe that $SH_2$-attached dyes really do not undergo any significant motion. First, the difference in the mean values of the angle $\theta$ in rigor and in the presence of ADP is so small in comparison with the standard deviation of this mean (Table IV, lines 8–10) that the difference between the means would have to be 2.9° (instead of the observed 1.8°) to be statistically important at the 50% confidence level according to a two-tailed $t$-test (Wonnacott & Wonnacott, 1985). In other words, while it is theoretically possible that the motion of $SH_2$ is such that it produces no change in fluorescent signal, in practice this would require that the induced motion had a component of less than 3° in the polar direction if the change in angle was to be undetected. We recognize the fact by choosing linear dichroism (LD) over fluorescence polarization (FP) we eliminated photobleaching at the expense of sacrificing the angular resolution. It is possible that by so doing we missed some features of probe orientation: Ajtai and Burghardt (1987) showed, for example, that FP, but not LD, could resolve changes in the orientation of 1,5-IAEDANS-labeled cross-bridge induced by MgADP. However, any angular reorientations of polar angle $\theta$ not resolved by our measurements would have to be smaller than 3°, because the lack of photobleaching allows us to measure fluorescence from the same area of the fiber with this precision (see above).

The second reason we consider that $SH_2$-bound probe does not undergo significant motion is that we have measured FP at three different wavelengths of the excitation light, thereby changing the direction that the probe absorption dipole makes with a reference frame fixed in S-1 (Ajtai & Burghardt, 1987). The chromophore was rhodamine, and the fluorescence was excited at 488 and 457.9 nm (in addition to 514.5 nm) by using the visible lines of argon laser. The result (Table IV) shows that MgADP was also ineffective in inducing any change in orientation of $SH_2$-bound probe at either of these wavelengths. Since the orientation of the absorption dipole of rhodamine must undergo a change as the wavelength decreases, the fact that there was no significant change in the dichroism at any wavelength following the addition of ADP supports the conclusion that there was no rotational movement in $SH_2$. However, although MgADP does not change the orientation of the region of S-1 containing $SH_2$, the environment of $SH_2$ does appear to be different in the presence of MgADP, because the $SH_2$ residue has a different reactivity in the presence of nucleotides (Yamaguchi & Sekine, 1966; Reisler, 1982; Wells

& Yount, 1980; Chaussepied et al., 1986).

We must also consider the possibility that $SH_2$-modified S-1 is inactive or that it cannot bind ADP. In fact, cross-linking $SH_2$ to $SH_1$ causes S-1 to lose most of its ATPase activity and binding to actin (due to the presence of nucleotide trapped in the active site; Wells & Yount, 1979, 1980). However, the following three results strongly support the view that $SH_2$-modified S-1 is still active and that it can bind ADP.

(1) S-1 with $SH_2$ modified or with $SH_1$ and $SH_2$ modified retains $Ca^{2+}$-ATPase activity (Table I) and its actin-binding ability (Figure 4) and can attach to actin filaments in the fiber in highly oriented fashion (Figure 5). Also, it has been shown by Reisler and Burke (1974) that myosin modified on $SH_2$ with NEM has $Ca^{2+}$-ATPase activity comparable to that of unmodified myosin, and therefore that it binds ATP (and therefore most likely also ADP). Another set of experiments suggests that MgADP binds to S-1 in which both $SH_1$ and $SH_2$ are modified (Lu et al., 1986): when S-1 is modified with the thiol-specific photoactivatable reagent benzophenone-4-iodoacetamide in the presence of MgATP, both $SH_1$ and $SH_2$ are labeled. When the photolysis of labeled S-1 is carried out, the reagent cross-links thiol groups to both 25- and 50-kDa tryptic fragments of S-1. There was a large difference in the extent of cross-linking when the photolysis was carried out in rigor as compared to when it was carried out in the presence of MgATP, suggesting that MgATP (and therefore most likely MgADP) binds to S-1 in which both $SH_1$ and $SH_2$ are modified. The following experiment similar to the one of Lu et al. (1986) also suggests that MgADP binds to S-1 in which both $SH_1$ and $SH_2$ are modified (Rajasekharan et al., 1987): when S-1 in which $SH_2$ was modified with a photoreactive derivative of 4,4′-bis(*N*-maleimido)benzophenone (MBP) is exposed to light, the reagent cross-links $SH_2$ to both 25- and 50-kDa tryptic fragments of S-1. This is a strong suggestion that S-1 modified with this reagent at $SH_2$ binds MgADP.

(2) $SH_2$-modified S-1 is still able to bind nucleotides since ATP makes all of it dissociate from actin, and $\epsilon$-ADP could bind the complex of actin and $SH_2$-modified S-1 with $K_a = 6 \times 10^3$ $M^{-1}$ (see Results). Reisler and Burke (1974) also reported that myosin with NEM on $SH_2$ has $Ca^{2+}$-ATPase activity comparable to that of unmodified myosin.

(3) A fluorescent label on $SH_1$ responded to MgADP regardless of whether $SH_2$ was modified or not (compare lines 1 and 7 in Table IV), and labels on both $SH_1$ and $SH_2$ respond to AMPPNP. This implies that S-1 modified at both $SH_1$ and $SH_2$ is active and can be used to study the motion of the region containing those thiols in response to nucleotides.

The dipole of IAR on $SH_1$ had an orientation of about 65° in rigor and 51° in the presence of ADP, while ATP and AMPPNP seemed to induce loosening of the actomyosin bond and randomization of the orientation. The orientation of the dipole of MLR on $SH_2$ was insensitive to ADP, but it was randomized by ATP and AMPPNP. The behavior of $SH_2$ is consistent with the observation by Craig et al. (1985) that the "arrowhead" appearance of actin filaments decorated with S-1 was destroyed by ATP but not by ADP.

In conclusion, our results show that two portions of S-1 respond differently to the addition of nucleotides, i.e., that S-1 is internally flexible. It is possible that this flexibility is associated with elasticity within the molecule and that the deformation of S-1 occurs during cross-bridge power stroke.

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**Registry No.** MgATP, 1476-84-2; MgADP, 7384-99-8; AMPPNP, 25612-73-1; Cys, 52-90-4.

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# Calmodulin and Troponin C Structures Studied by Fourier Transform Infrared Spectroscopy: Effects of $Ca^{2+}$ and $Mg^{2+}$ Binding†

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ABSTRACT: Fourier transform infrared (FTIR) spectroscopy has been used to examine the conformationally sensitive amide I′ bands of calmodulin and troponin C. These are observed to undergo a sequence of spectroscopic changes which reflect conformational rearrangements that take place when $Ca^{2+}$ is bound. Calmodulin and troponin C show similar though not identical changes on $Ca^{2+}$ binding, and the effect of $Mg^{2+}$ on troponin C is quite different from that of $Ca^{2+}$. Both proteins show absorption maxima in the amide I′ region at 1644 $cm^{-1}$ which is significantly lower in frequency than has been generally observed for proteins that contain a high percentage of $\alpha$-helix. It is proposed that an unusually high proportion of the helices in the structures of these proteins are distorted from the normal $\alpha$-helical configuration such that the carbonyl stretching frequencies are lowered. It is further proposed that the shift to lower frequency is due to backbone carbonyl groups in the distorted helices that form strong hydrogen bonds with solvent molecules. A decrease in intensity at 1654 $cm^{-1}$, the normal frequency assignment for $\alpha$-helical structure, is observed as $Ca^{2+}$ binds to calmodulin and troponin C. This suggests that $Ca^{2+}$ binding results in a net decrease in "normal" $\alpha$-helix conformation. There is a corresponding increase in intensity of the band at 1644 $cm^{-1}$, possibly due to an increase in distorted helix content, allowing for a net increase in helix consistent with circular dichroism estimates of the $Ca^{2+}$-dependent changes in helix content in calmodulin.

Calmodulin is a ubiquitous $Ca^{2+}$ binding protein that regulates a wide variety of cellular functions including cyclic nucleotide phosphodiesterase (Dedman et al., 1977; Huang et al., 1981; Cox et al., 1981), adenylate cyclase (Valverde et al., 1979), phosphorylase kinase (Cohen, 1980), and myosin light chain kinase (Walsh et al., 1979; Bartelt et al., 1987). Troponin C is the $Ca^{2+}$ binding protein involved in regulation of muscle contraction (Herzberg et al., 1986). Troponin C and calmodulin are thought to be evolutionarily related since they show a high degree of structural homology and can cross-react in their respective biochemical systems [see Means and Dedman (1980) and references cited therein]. They belong to a larger class of structurally related low molecular weight $Ca^{2+}$ binding proteins that also includes parvalbumin.

Both calmodulin and troponin C bind up to four $Ca^{2+}$ ions. In calmodulin, $Ca^{2+}$ binding results in an increase in radius of gyration and a lengthening of the molecule (Seaton et al., 1985; Heidorn & Trewhella, 1988) which is accompanied by an increase in $\alpha$-helix content, as estimated by a variety of spectrscopic techniques. These changes result in the exposure of hydrophobic regions of the molecule that are thought to be important in interactions with target enzymes (Burger et al., 1984). The conformational changes induced by $Ca^{2+}$ binding to troponin C result in a shift in the position of tropomyosin, allowing contact between myosin and the actin thin filament.

The crystal structures of both calmodulin (Babu et al., 1985) and troponin C (Herzberg & James, 1985; Sundaralingam et al., 1985; Satyshur et al., 1988) have been solved. In both cases, the proteins were crystallized in the presence of excess $Ca^{2+}$ and at low pH (approximately 5.5). The calmodulin structure was determined with all four $Ca^{2+}$ sites occupied, while in the case of troponin C only two of the four sites were occupied. Both proteins showed unusual "dumbbell" structures consisting of two globular domains at opposite ends of an interconnecting $\alpha$-helical segment which is largely exposed to solvent and makes few contacts with the rest of the molecule. The globular domains contain two $Ca^{2+}$ binding sites each and show a high degree of structural homology with each other and with parvalbumin. The $Ca^{2+}$ binding sites have a helix-loop-helix structural motif which is widely conserved among

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