Kuttner, T., & Lichtenstein, L. (1930) *J. Biol. Chem. 86*, 671–686.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Li, H. C., Hsiao, K. J., & Chan, W. W. S. (1978) *Eur. J. Biochem. 84*, 215–225.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Martonosi, A., & Feretos, R. (1964) *J. Biol. Chem. 239*, 648–668.
Moeschler, H. J., Malencik, D. A., Pocinwong, S., Alaba, O., Kerrick, G. L., & Fischer, E. H. (1979) *Biochimie 61*, 615–624.
Moss, J., Manganiello, V. C., & Vaughan, M. (1978) *Biochim. Biophys. Acta 541*, 279–287.
Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691–5697.
Reimann, E. M., & Umfleet, R. A. (1978) *Biochim. Biophys. Acta 523*, 516–521.
Rubin, C. S., Erlichman, J., & Rosen, O. M. (1974) *Methods Enzymol. 38*, 308–315.
Schwartz, A., Entman, M. L., Kaniike, K., Lane, L. K., Winkle, W. B. V., & Bornet, E. P. (1976) *Biochim. Biophys. Acta 426*, 57–72.
Spector, T. (1978) *Anal. Biochem. 86*, 142–146.
Tada, M., Ohmori, F., Yamada, M., & Abe, H. (1979) *J. Biol. Chem. 254*, 319–326.
Teo, T. S., Wang, T. H., & Wang, J. H. (1973) *J. Biol. Chem. 248*, 588–595.
Varsányi, M., Gröschel-Stewart, U., & Heilmeyer, L. M. G., Jr. (1978) *Eur. J. Biochem. 87*, 331–340.
Walsh, M., & Stevens, F. C. (1978) *Can. J. Biochem. 56*, 420–429.
Walsh, M., Vallet, B., & Demaille, J. G. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 2952a.
Will, H., Blanck, J., Smettan, G., & Wollenberger, A. (1976) *Biochim. Biophys. Acta 449*, 295–303.
Wray, H. L., & Gray, R. R. (1977) *Biochim. Biophys. Acta 461*, 441–459.
Wray, H. L., Gray, R. R., & Olsson, R. A. (1973) *J. Biol. Chem. 248*, 1496–1498.

Consequences of Reacting the Thiols of Myosin Subfragment 1†

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ABSTRACT: When subfragment 1 (S-1) of myosin is exposed (120 h at 0 °C) to a series of increasing concentrations of methyl methanethiosulfonate (MMTS) up to 25 × [S-1], the accessible thiols of S-1 are progressively reacted to form $-SSCH_3$. If "SH_1", the more readily reactive thiol of S-1, is first labeled with the fluorescent dye 1,5-IAEDANS [*N*-(iodoacetyl)-*N*-(5-sulfo-1-naphthyl)ethylenediamine], seven additional thiols can be reacted with MMTS. In 8 M urea 12 thiols of S-1 are titrated with PCMB [*p*-(chloromercuri)benzoate]. Thus, four thiols are considered to be "inaccessible" to MMTS under the exposure conditions used. S-1 ATPase activities (Ca^{2+}, Mg^{2+}, and actin activated) decline as more MMTS is bound but do not fall to zero even at [MMTS]/[S-1] ratios up to 100:1. With fluorescent-labeled S-1, the affinity of S-1 for F-actin is measured by following the time-resolved fluorescence anisotropy decay. As more MMTS is bound to S-1, the affinity constant decreases from about 10 μM^{-1} to about 2 μM^{-1} (25 °C; 0.284 M KCl, 1.35 mM $MgCl_2$, and 0.45 mM EGTA, buffered at pH 7.4). In the presence of ATP there is no measurable affinity. Since reacting S-1 thiols with a small blocking group such as MMTS does not abolish S-1 ATPase activity or actin binding, it is concluded that thiols are not directly involved in these functions. The effects of reacting S-1 thiols with MMTS may be attributed to conformational changes in S-1. To test this hypothesis, we applied certain probes of structural changes at different stages of blockage with MMTS. The fluorescence of 8-anilino-1-naphthalenesulfonate equilibrated with the system increased with increased blockage but not hand in hand with the ATPase activity. However, signals (emanating from 1,5-IAEDANS placed at thiol SH_1) from two other probes correlated very well with loss of ATPase activity and with loss of actin affinity, and their behavior can be taken to mean that certain structural changes are progressively caused by progressive blockage. The rotational correlation time of the S-1 particle increases, suggesting a progressive global deformation. The magnitude of the excited-state lifetime decreases upon ATP addition, suggesting a progressive accessibility of solvent to the region immediately near to SH_1.

Pursuing our laboratory's long-standing interest in the possibility that "activation" and "inhibition" (collectively, "modification") of myosin ATPase are conformational phenomena rather than a consequence of thiol reaction per se (Rainford et al., 1964; Hartshorne & Morales, 1965), we undertook a study of the effects of reacting myosin thiols using a "small" SH reagent, with the idea of minimizing *indirect* effects of thiol reaction, e.g., results of putting bulky groups at structurally sensitive locations. Our experiments were designed to examine also the essentiality of thiols to the myosin affinity for actin. Finally, we included in our design optical probes that might sense conformational changes if these occurred. The consequences of reacting the thiols of myosin S-1[1]

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[1] Abbreviations used: S-1, myosin subfragment 1; SH_1, fast-reacting thiol in S-1; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*-(5-sulfo-1-naphthyl)-ethylenediamine; MMTS, methyl methanethiosulfonate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PCMB, *p*-(chloromercuri)benzoate; DTT, dithiothreitol; Ans, 8-anilino-1-naphthalenesulfonate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; IAA, iodoacetamide.

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are reported in terms of the changes in ATPase activities and in the affinity of S-1 for F-actin. The latter is measured by time-resolved fluorescence anisotropy decay. When all the accessible thiols are blocked by MMTS, the two major functions of S-1, ATPase activity and actin binding, are strongly affected but persist at detectable levels. These results support the view that (a) accessible free thiols are inessential for these particular functions (of course, the thiols may serve other roles not yet discovered) and (b) placing groups at thiol locations affects the functions indirectly, presumably by causing structural ("conformational") changes.

The idea that modification results from an indirectly produced conformational change and not because thiols per se are reacted has been advanced not only by colleagues here but also independently by Tonomura et al. (1961), as well as by Kielley and his associates (Sekine & Kielley, 1964). However, the strategy of trying to eliminate direct thiol involvement, by asking whether the myosin functions persist after thiol reaction, was pioneered by Bárány and his associates (Gaetjens et al., 1964). Also, as this paper was readied for publication, there appeared a very interesting and closely related effort by Eckstein and his associates (Wiedner et al., 1978). We believe that the qualitative similarities in the conclusions of these two groups with our own are more important than the substantial quantitative differences among the three.

The occurrence of conformational changes is tested for by various methods. In the first method we look for the extent to which 8-anilino-1-naphthalenesulfonate (Ans) penetrates and binds to S-1; this extent is taken to be proportional to the fluorescence emitted by the bound Ans (Weber & Young, 1964). Previously, our laboratory introduced this method to follow the *p*-(chloromercuri)benzoate (PCMB) and ethylenediaminetetraacetate (EDTA) titration of myosin (Duke et al., 1966) and also to follow changes in myosin with age or temperature (Lim & Botts, 1967). The second and third methods depend on uniquely and covalently attaching the fluorescent dye *N*-(iodoacetyl)-*N*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) (Takashi et al., 1976) to the most reactive thiol of S-1, viz., to "SH_1". It has been shown (Mendelson et al., 1973, 1975) that in this attachment the fluorophore is immobilized in a presumably hydrophobic environment (excited-state lifetime $\tau \simeq 20$ ns). In the second method advantage is taken of the immobilization; from study of the time-resolved fluorescence anistropy decay of the imbedded dye, the rotational correlation time Φ of the S-1 molecule is deduced (Mendelson et al., 1973); changes in volume or asymmetry of S-1 might be expected to be expressed as changes in the parameter Φ. In the third method the time-resolved fluorescence anisotropy decay data are used to compute the decay in total fluorescence intensity; from this decay is calculated the excited-state lifetime, τ, of the attached dye. Because dielectric relaxation through accessibility to water is an effective way of shortening τ, changes in conformation near the attached dye, i.e., near SH_1, might be expected to cause changes in τ.

Materials and Methods

Proteins. Myosin was extracted from the back muscle of rabbits according to Tonomura et al. (1966). S-1 was prepared from fresh myosin by digesting with insoluble papain in the presence of Mg^{2+} according to a modified method of Lowey et al. (1969). The conditions for papain digestion of the insoluble myosin solution were 10 mM Hepes, 2 mM $MgCl_2$, 40 mM KCl, and 2 mM DTT, pH 7.0. After chromatography (Sephadex G-200) in which the S-1 was purified and DTT was removed, the S-1 solution was kept under N_2 to prevent cysteine oxidation. Also, in our case the papain concentration was predetermined by an activity measurement (0.06 unit/mL) and the proteolysis was stopped, after centrifugation, by N^{α}-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride. F-actin was prepared by using the method of Spudich & Watt (1971). Free ATP was removed from F-actin for time-resolved fluorescence anisotropy decay measurements by use of Dowex AG 1-X4 resin. Papain was purchased from Worthington Biochemicals.

Protein Concentration. S-1 concentration was measured by absorbance at 280 nm ($\epsilon_{280} = 8.9 \times 10^4$ M^{-1} cm^{-1}) (Young et al., 1965). Actin concentration was measured by absorbance at 290 nm (G-actin, $\epsilon_{290} = 2.71 \times 10^4$ M^{-1} cm^{-1}) (Houk & Ue, 1974). In each case the appropriate correction for light scattering was applied. The molecular weights used were 115 000 for S-1 (Lowey et al., 1969) and 42 000 for G-actin (Elzinga et al., 1973).

Chemicals. [^{3}H]-1,5-IAEDANS (16.6 mCi/mmol) was synthesized by R. H. Haugland in our laboratory according to the procedure of R. H. Fairclough (private communication). [^{14}C]Methyl methanethiosulfonate (MMTS; 0.049 mCi/mmol) was a gift from Dr. S. Currier of Tufts Medical School's Biochemistry Department. ATP was purchased from Sigma (99% assay). All other chemicals were reagent grade.

Labeling of S-1. Freshly prepared S-1 (50–100 μM) was incubated with a 1:1 molar ratio of [^{3}H]-1,5-IAEDANS in a buffer solution of 0.15 M KCl, 10 mM Hepes, and 5 μM TLCK, pH 7.5, at 0 °C for 24 h. S-1 (20 μM) was incubated with various amounts of [^{14}C]MMTS in the same buffer solution at 0 °C for various lengths of time. The labeling with [^{14}C]MMTS was also carried out under identical conditions as described above with the inclusion of 1 mM MgADP.

Determination of Stoichiometry of Labeling. After the appropriate reaction time, 1.5 mL of the incubation solution (S-1, label, 0.15 M KCl, 10 mM Hepes, and 5 μM TLCK, pH 7.5) was applied to a Sephadex G-25 column (prewashed with KCl–Hepes buffer) in order to remove excess label. After protein concentration determination, an aliquot of the protein solution was added to 1 mL of scintillator cocktail [85% enconofluor (NEN) and 15% BBS-3 (Beckman)]. Radioactivity of the vials was determined in a Beckman LS-150 scintillation counter.

Titration of Free Sulfhydryl Groups by PCMB. Titration of free sulfhydryl groups on S-1 was carried out with PCMB via a modified procedure of Boyer (1954). For titrating the total number of thiols, we denatured the S-1 in an 8 M urea solution before titration.

Determination of ATPase Activity. The hydrolysis of ATP by S-1 in the presence of Ca^{2+} was measured by the method of Fiske & Subbarow (1925); Mg^{2+} and actin-activated Mg^{2+}–ATPase were measured by the method of Lin & Morales (1977). At low actin-activated Mg^{2+}–ATPase values, we corroborated our results by the method of Fiske & Subbarow. Standard assays were performed with magnetic stirring at 25 °C and pH 7.5 for periods of 2–30 min. Mg^{2+}–ATPase: 11 mM Hepes, 50 mM KCl, 1.3 mM $MgCl_2$, and 1.3 mM ATP. Ca^{2+}–ATPase: 9 mM $CaCl_2$, 0.9 mM ATP, 0.56 M KCl, and 23 mM Hepes. Actin-activated Mg^{2+}–ATPase: 1.3 mM ATP, 63 mM KCl, 1.3 mM $MgCl_2$, and 11 mM Hepes; [actin]/[S-1] = 3–4.

Labeling of S-1 with Iodoacetamide. The reactive thiol of S-1 was specifically labeled with iodoacetamide (IAA) as described by Takashi et al. (1976) in the labeling of myosin. S-1 (80 μM) was incubated with a 40-fold molar excess of IAA in 0.6 M KCl and 10 mM Tes, pH 7.0, at 0 °C. After incu-

bation for 2 h, 60% saturated ammonium sulfate solution was added to the S-1 solution to stop the labeling. The S-1 precipitate was collected by centrifugation at 9000*g* for 20 min. This was dissolved in 15 mL of buffer solution (0.15 M KCl and 10 mM Hepes, pH 7.5) and then dialyzed against the same buffer for several days to remove ammonium sulfate. As evidence of ATPase activation due to SH_1 labeling, the Ca^{2+}–ATPase activities of unlabeled and labeled S-1 were measured and found to be 0.86 and 5.06 s^{-1}, respectively.

Steady-State Measurement of the Fluorescence When Ans Is Reacted with S-1·IAA. Change of fluorescence intensity after addition of Ans to IAA-labeled S-1 was measured at 0 °C on a Hitachi Perkin-Elmer MPF-4 spectrofluorometer used in the ratio mode and equipped with a temperature-controlled cell holder. The wavelength 380 nm was used for excitation and 475 nm was used for emission. The effective slit widths on the excitation and emission monochromators were 10 and 2 nm, respectively.

Time-Resolved Fluorescence Measurements of 1,5-IAEDANS-Labeled S-1. These measurements were made on a noncommercial "double-beam" photon-counting apparatus (Mendelson et al., 1975) that records the two orthogonally polarized intensities ($I_{\parallel}$ and $I_{\perp}$) of the fluorescence emitted by the solution as a function of time, t. From these values the total intensity, $I_{tot}(t) = I_{\parallel} + 2I_{\perp}$, and the anisotropy, $r(t) = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$, are obtained. The time required for $I_{tot}(t)$ to decay to $1/e$ of its initial value is taken as the excited-state lifetime, τ, of the fluorophore. Normally, about 2×10^7 photons are counted over a period of about 30–40 min. On the nanosecond time scale of the present experiments, the fluorophore has been shown to be immobile relative to the protein (Mendelson et al., 1973). Therefore, the time that it takes $r(t)$ to decay to $1/e$ of its initial value is taken as the rotational correlation time, Φ, of the S-1 particle. It was originally shown by Mendelson et al. (1973) that, in the case of S-1 labeled at its most reactive thiol, $r(t)$ can be represented as a single exponential, and it was argued that therefore $\Phi = (6D_{rot})^{-1}$, where D_{rot} is the rotational diffusion coefficient for end-over-end tumbling of S-1 [by taking S-1 to be elongate; see Kretzschmar et al. (1978)].

In some experiments we deal with binary mixtures of S-1, e.g., n_1 moles of free and n_2 moles of bound. In such cases it is assumed that the fluorophores emit independently. Then, the specific molar total intensity (what is observed for the mixture as I_{tot} divided by the total number of moles) is a linear function of the mole fractions x_1 and x_2; i.e.

$$I_{tot}(t)/(n_1 + n_2) = x_1 i_{tot}^{1}(0)e^{-t/\tau_1} + x_2 i_{tot}^{2}(0)e^{-t/\tau_2} \quad (1)$$

where τ_1 and τ_2 are the corresponding excited-state lifetimes, and the i's are the molar fluorescences of the components. The anisotropy, on the other hand, is not, in general, a linear function of the mole fractions. But it becomes so if the individual total intensities ($I_{\parallel} + 2I_{\perp}$) are not too different [the differences, studied in the present and in related work, being of the order of 5% (Mendelson et al., 1973)]. If, further, $r_1(0) - r_2(0) = r(0)$, then

$$\ln r(t) = \ln r(0) + \ln (x_1 e^{-t/\Phi_1} + x_2 e^{-t/\Phi_2}) \quad (2)$$

and

$$\frac{d \ln r(t)}{dt} = \frac{(x_1/\Phi_1)e^{-t/\Phi_1} + (x_2/\Phi_2)e^{-t/\Phi_2}}{x_1 e^{-t/\Phi_1} + x_2 e^{-t/\Phi_2}} \quad (3)$$

The fitting procedure when 1 and 2 refer respectively to free and actin-bound S-1 is as follows. Equation 2 represents a curve whose instantaneous derivative with respect to time is given by eq 3. For short time intervals, the curve $\ln r(t)$ is well approximated by a straight line. For example, if $b - a$ is small, then in the interval from $t = a$ to $t = b$

$$\ln r_{a,b}(t) = \ln r_{a,b}(0) - (1/\Phi_{a,b})t \quad (4)$$

The parameter $1/\Phi_{a,b}$ is evaluated by fitting the line eq 4 to the data points for $\ln r(t)$ in the time interval ab, using least-squares techniques. The slope of the line, $(1/\Phi_{a,b})$, can be equated to the right-hand side of eq 3 if the latter is evaluated at the "right" time, say t_m. Since the counting rate decreases with increasing t, the slope measurement depends more heavily on the earlier portion of the time interval. The time t_m corresponding to this measured slope was taken to be that giving the same number of photon counts between a and t_m as between t_m and b. (Actually, the choice of t_m within the time interval used here, 37–72 ns, is not critical.) With t_m evaluated, x_1 becomes the only unknown in the equation since $x_2 = 1 - x_1$, Φ_1 is known from measurements on S-1 in the absence of actin, and Φ_2 is essentially infinite (assumed to be 10^5 ns).

Once x_1 is known, the equilibrium constant between forms 1 and 2 is calculated as

$$K = [(1 - x_1)/x_1]/[A_0 - (1 - x_1)M_0] \quad (5)$$

where A_0 and M_0 are respectively the total molar concentrations of actin and S-1. This general method of measuring affinity constants among associating muscle proteins has been discussed elsewhere by one of us (Botts et al., 1975) and by Highsmith et al. (1976), but the foregoing analysis has not been prevously given. In the latter paper there is presented evidence that the labeling of S-1 at its reactive thiol has no perceptible effect on K.

Time-resolved fluorescence anisotropy decay measurements were made at 25 °C, pH 7.4. The solvent was 0.284 M KCl–13 mM Hepes buffer, 1.35 mM $MgCl_2$, 0.45 mM EGTA, and 0.56 μM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride. K is known to decrease with increasing ionic strength, so the absolute values of K reported here are lower than they would be had, say, 0.15 M KCl been used; however, our purpose was to study changes on modification, and K is more precisely measured when its value is in a range lower than "physiological". The concentrations of S-1 and actin (as monomer) were 0.2 and 0.65 μM, respectively. The time at which actin stood at this concentration was minimized so as to avoid depolymerization.

Results

One goal in this work has been to study the effect of blocking thiols on the actin-activated Mg^{2+}–ATPase and on the actin affinity of S-1 because these are the functions closely related to muscle contraction. Since usually we wished to make parallel studies on the ATPase activity and the actin affinity of S-1 and since our measurement of affinity involves a fluorescent probe covalently attached at the "SH_1" thiol of S-1 (Takashi et al., 1976), we were typically studying the effects of reacting thiols other than SH_1 with MMTS (Smith et al., 1975). However, studies made in the absence of the probe showed that asymptotically, i.e., with increasingly severe exposures to MMTS (120–168-h incubation with [MMTS]/[S-1] up to 25) the various ATPases behaved no differently from how they behaved when the probe was attached to SH_1 (Figure 1). In particular, it is seen that Mg^{2+}–ATPase is far from being extinguished though obviously it is decreasing. In this respect we confirm the results of Gaetjens et al. (1964). Wiedner et al. (1978) reported a monotonic increase under similar conditions. Also, we found the other ATPases to be decreasing more precipitously at high exposures, whereas

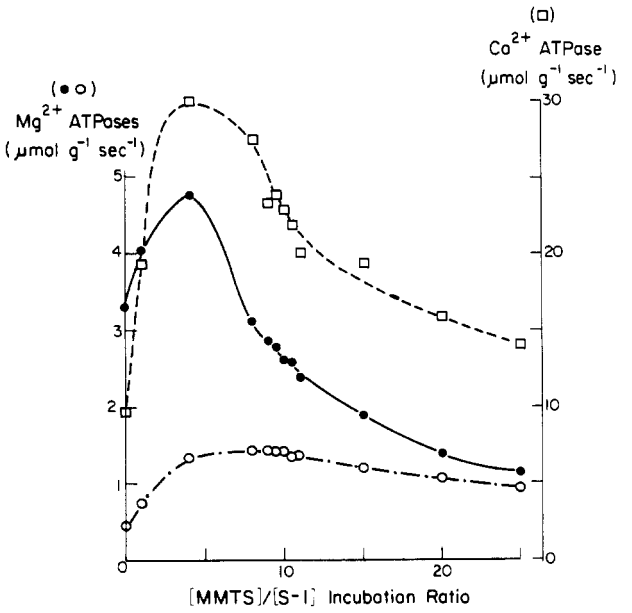

FIGURE 1: ATPase activities of S-1 as a function of incubation ratio with MMTS. (□) Ca^{2+}–ATPase; (●) actin-activated Mg^{2+}–ATPase; (○) Mg^{2+}–ATPase. Assays are as described under Materials and Methods.

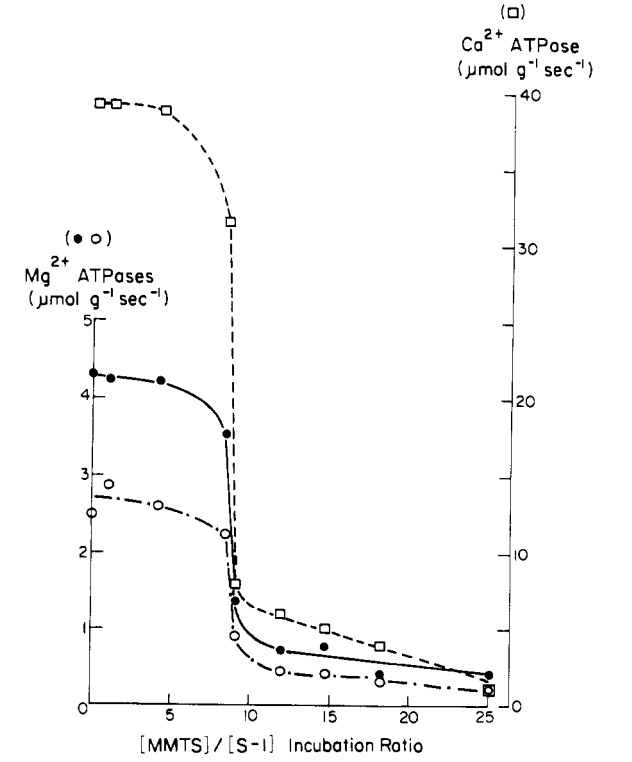

FIGURE 2: ATPase activities of 1,5-IAEDANS-labeled S-1 as a function of incubation ratio with MMTS. (□) Ca^{2+}–ATPase; (●) actin-activated Mg^{2+}–ATPase; (○) Mg^{2+}–ATPase. Assays are as described under Materials and Methods.

Wiedner et al. (1978) reported that their Ca^{2+}–ATPase was progressively increasing at high exposures.

Figure 2 indicates that prereaction of the S-1 with 1,5-IAEDANS (specific for SH_1) sharpens the decrease of ATPase activity with MMTS exposure (labeling with radioactive 1,5-IAEDANS showed [bound 1,5-IAEDANS]/[S-1] = 0.73).

When SH_1 is specifically labeled with 1,5-IAEDANS and the S-1 preparation is then exposed to various concentrations of MMTS for 120 h, the number of thiol groups reacted with MMTS increases asymptotically to about 7 mol/mol of S-1

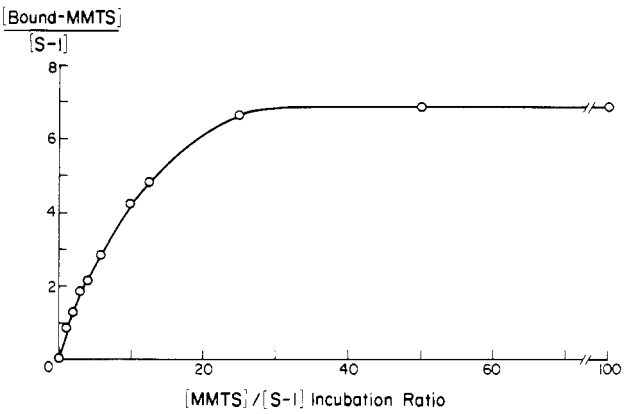

FIGURE 3: Moles of MMTS reacted per mole of S-1 (previously exposed to 1,5-IAEDANS in 1:1 ratio) as a function of incubation ratio with MMTS. Reaction time, 120 h at 0 °C; 0.15 M KCl, 10 mM Hepes, 5 μM TLCK, and 20 μM S-1.

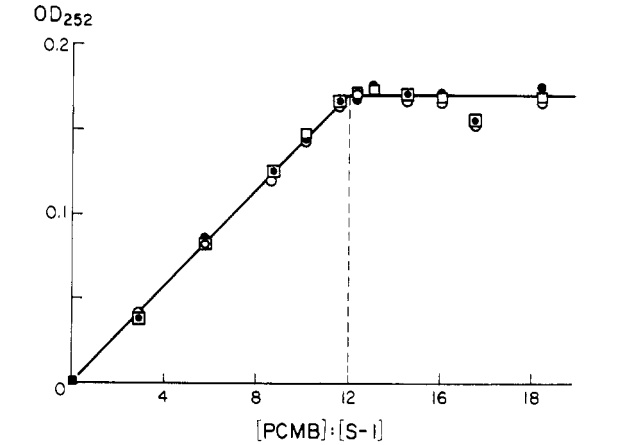

FIGURE 4: PCMB titration of S-1: 2.86 μM S-1; asymptotic level of [bound PCMB] = 34.20 μM or 11.96 × [S-1]. Conditions: 8 M urea, 40 mM Hepes, and 6 mM KCl; incubation time at room temperature (□) 3.5, (●) 21.5, and (○) 46.5 h.

(Figure 3), which is to say that the *total* number of thiols reacted levels off at 8/mol of S-1. This number is not changed if the MMTS exposure is conducted while the S-1 is in equilibrium with ADP. The amino acid analysis of S-1 (Lowey et al., 1969) shows that S-1 contains slightly over 12 thiols, so a question arises regarding the state of the missing 4 thiols. But, by direct titration with PCMB in the presence of 8 M urea, we found essentially all 12 thiols (Figure 4) so we conclude that, under our conditions of exposure, MMTS reacts with all the accessible thiols (in the absence or presence of nucleotide). We also conclude that, after exposure to MMTS, the remaining thiols fail to react because the protein structure denies access to the reagent, not because they have formed disulfides or other oxidation products (Weshaus & Pohl, 1978).

Figure 5 shows that as the accessible thiols are titrated both the actin-activated ATPase and the affinity constant for binding to actin diminish more or less in parallel (although the population of molecules being monitored is not identical in the two cases since the ATPase measurements include the contribution of the *un*labeled enzyme whereas the time-resolved anisotropy decay instrument senses only the labeled protein). It is seen that the asymptotic levels in Figure 5 are both nonzero. This is obvious for the actin affinity, but, because the ATPase activity drops to only about 7% of its initial level, the residual activity was reexamined after more drastic exposure to MMTS. We found that even after a 120-h exposure to a 100-fold excess of MMTS enzymatic activity was still

detectable. For Figure 5 it is also clear that in the presence of ATP the affinity for actin is zero regardless of the extent of modification by MMTS. From the foregoing results we conclude that, even after all the accessible thiols of S-1 have been reacted with a small blocking group, there persist nonzero levels of affinity for actin and of various ATPases (including the actin activated). At the same time, it is evident that blocking of seven thiols in addition to SH_1 certainly has an effect—in some cases a profound effect—on all the S-1 functions studied.

Using myosin instead of S-1, we had previously found a rather sharp "transition" increase in Ans fluorescence as the protein was titrated with PCMB. The extent of thiol reaction at which this "transition" occurred seemed to be that at which the Ca^{2+}–ATPase activity fell most rapidly (Duke et al., 1966) (cf. Figure 2). It was surmised at the time that Ans fluorescence was sensing the structural changes that brought about the decrease in activity. In this work we have found that Ans fluorescence probably senses not only these changes but also others as well; as a result, the major decrease in activity and increase in Ans fluorescence are largely separable in time. Figure 6 shows experiments using S-1 whose SH_1 has been blocked with IAA to simulate blocking with 1,5-IAEDANS (since fluorescence of the latter would confuse the measurement of Ans fluorescence). For incubation with various molar ratios of MMTS to S-1, actin-activated Mg^{2+}–ATPase and Ans fluorescence are shown as functions of *incubation time* with MMTS. It is seen that the greater the MMTS/S-1 incubation ratio the greater the *asymptotic* level of Ans fluorescence and the smaller the *asymptotic* level of ATPase activity. At all ratios of MMTS/S-1, the fall[2] in activity appears to precede the rise in fluorescence. This separation of behaviors is brought out most clearly in a "phase plot" (Figure 6B). In this plot the history of an individual sample (a single MMTS/S-1 incubation ratio) is a path of head-to-tail arrows, each arrow showing the changes from one measurement time to the next. Along any path the lengths of the arrows (magnitudes of the changes) progressively decrease because each system is reaching an equilibrium. As illustrated with the lowest [MMTS], the components of the arrows show that early changes consist of large decreases in ATPase activity and small increases in fluorescence. For the two highest [MMTS], the decrease in activity is so rapid that it is almost complete before the first time point, whereas the fluorescence continues to increase for a considerable time thereafter. So, changes in activity are not tightly coupled in time with changes in fluorescence.

Figure 7 shows the situation after a 120-h incubation of 1,5-IAEDANS-labeled S-1 with MMTS. Plotted against n, the number of reacted thiols per mole of S-1, are two parameters of free S-1, Φ and τ, which might be expected to reveal structural changes. As dashed lines we have also shown the corresponding actin-activated Mg^{2+}–ATPase and actin affinity constant (repeated from Figure 5). In the absence of ATP, it is evident that Φ and τ do not change very much as the thiols of S-1 are progressively reacted. On the other hand, in the presence of ATP, thiol reaction causes significant increases in Φ and decreases in τ. These changes, unlike those in Ans fluorescence, do go hand in hand with the decrease in enzymatic activity and actin affinity.

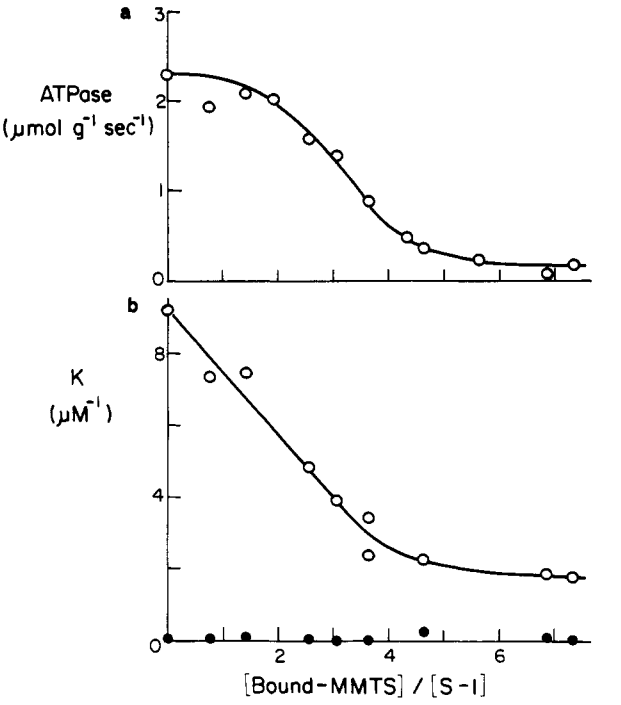

FIGURE 5: (a) Actin-activated Mg^{2+}–ATPase activity as a function of MMTS bound to 1,5-IAEDANS-labeled S-1. Standard assays (3–30 min): 1.3 mM MgATP, 63 mM KCl, and 11 mM Hepes; [actin]/[S-1] = 3–4, at 25 °C and pH 7.5. (b) Actin affinity of 1,5-IAEDANS-labeled S-1 as a function of MMTS bound to this S-1. (○) No ATP; (●) 4.8 mM ATP.

Discussion

One of the questions posed in this work was what happens to the major functions of S-1—ATPase activity and actin-binding ability—as the thiols of S-1 are progressively caused to disappear? Operationally, what we did in this work to "cause a thiol to disappear" was to attach to it a *small*, neutral blocking group; we found that without first denaturing S-1 we could not do this to *all* the thiols, but we could progressively react the (8 out of 12) "accessible" thiols. The answer to the question is that, as these accessible thiols disappear, ATPase and actin binding *diminish but do not disappear*. Therefore, none of these thiols can be playing a *direct* role in either process (this is not to say that thiols are inessential in other respects). As regards enzymatic activity, this conclusion is in complete accord with Gaetjens et al. (1964); qualitatively, it is also in accord with Wiedner et al. (1978) despite some quantitative differences between their results and ours. As regards affinity for actin, Gaetjens et al. (1964) found no effect of thiol blockage; we find that progressive disappearance of thiols definitely reduces the actin affinity, but the asymptotic level to which it is reduced is far from zero. The discrepancy in this comparison is apt to stem from sensitivity of method; we used S-1 rather than myosin and had the advantage of the recently developed time-resolved fluorescence anisotropy decay method for sensing binding.

[2] For the ratio 4, the activity initially rises. This is presumably due to the initial presence of some free SH_1; it is hard to label with iodoacetamide at precisely 1:1, and in this case the labeling ratio was apparently slightly less than 1:1. Above, it was convenient to classify 12 thiols of S-1 into approximately 8 accessible and 4 inaccessible thiols, on the basis of reaction with MMTS and PCMB. It was pointed out, however, that this is not a rigorous assignment and that, given enough time, both reagents—especially PCMB—slowly react with the "inacessible" thiols after the "accessible" thiols are blocked (unpublished data). Here we have found that the "asymptotic" levels in ANS fluorescence reached after a very long time are greater the greater the [MMTS]/[S-1] incubation ratio. It is suggested that this phenomenon may be a manifestation of the slow penetration by the thiol reagent into the inaccessible thiols, with a resulting exposure of previously recessed regions of the S-1. This sort of change may destroy the enzymatic site, so that the asymptotic level of ATPase reached after an extended time would also depend on the [MMTS]/[S-1] ratio.

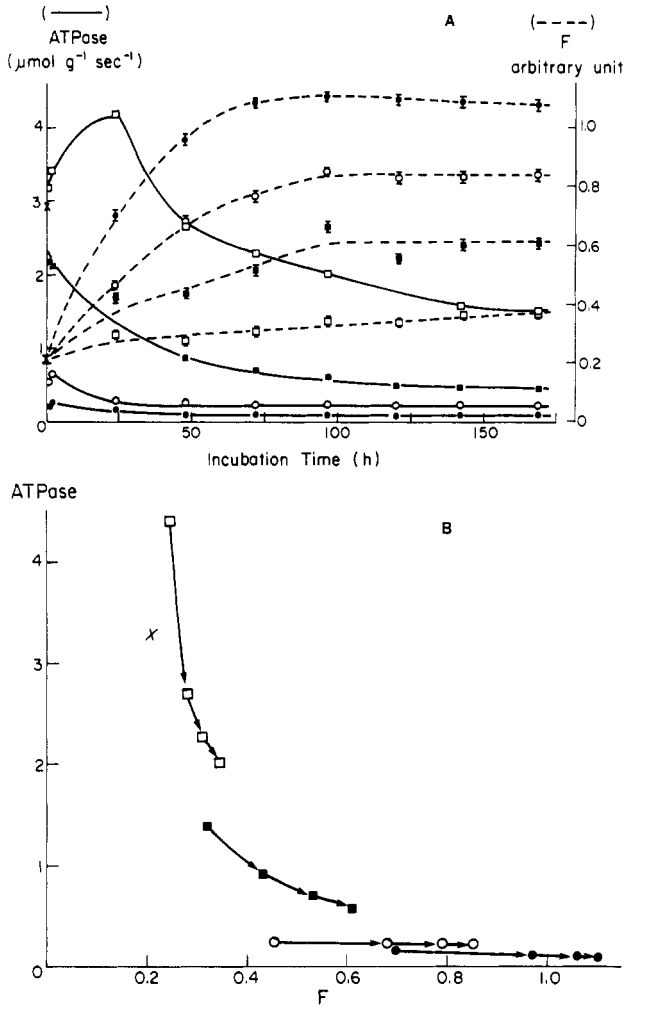

FIGURE 6: (A) Actin-activated Mg^{2+}–ATPase activity and Ans fluorescence of IAA-labeled S-1 as a function of incubation ratio with MMTS. Conditions: (—) actin-activated Mg^{2+}–ATPase (1.3 mM ATP, 63 mM KCl, 1.3 mM $MgCl_2$, and 11 mM Hepes; [actin]/[S-1] = 3–4, at 25 °C and pH 7.5); (- - -) Ans fluorescence (1.3 μM S-1, 60 mM KCl, 20 mM Hepes, and 6.5 μM Ans, at 0 °C and pH 7.5). [MMTS]/[S-1]: 4 (□); 12.5 (■); 25 (○); 50 (●). (B) Phase plot of ATPase activity and Ans fluorescence replotted from smoothed curves in (A). X: IAA-labeled S-1 control.

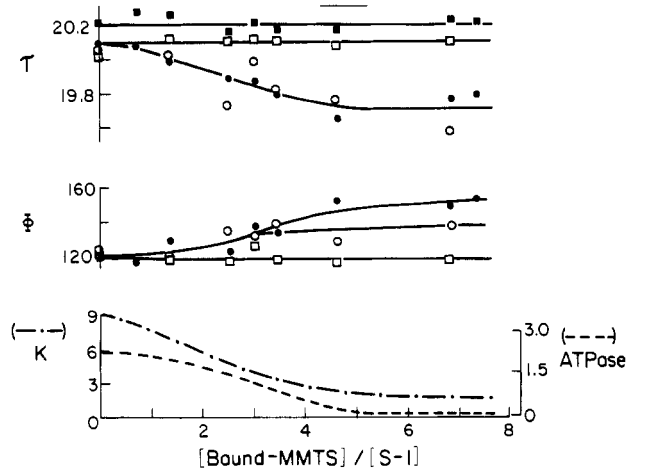

FIGURE 7: Excited-state lifetime (τ) and rotational correlation time (Φ) for 1,5-IAEDANS-labeled S-1 as a function of MMTS bound to S-1. Experimental conditions are described under Materials and Methods. (□) S-1; (■) S-1 + F-actin; (○) S-1 + ATP; (●) S-1 + F-actin + ATP. The lowest graph shows the corresponding actin-activated Mg^{2+}–ATPase and actin-affinity constant repeated from Figure 5.

That thiol blockage affects ATPase considerably more than it affects affinity for actin is another indication that the two sites on S-1 are spatially distinct. This again is a concept first advanced by Bárány & Bárány (1959) and recently proved unequivocally by the existence of actin–S-1–nucleotide complexes (Beinfeld & Martonosi, 1975; Highsmith, 1976). It is also of interest that even in heavily modified S-1 addition of ATP still causes total dissociation of actin, indicating that whatever mechanism conveys information from the nucleotide site to the actin site remains operative.

Progressive blocking of the accessible thiols may not abolish the ATPase and actin binding of S-1, but it certainly impairs both functions. What is the mechanism of this impairment? Because thiol-unrelated reagents cause the *same* impairment, others (Tonomura, 1961; Sekine & Kielley, 1964) and our colleagues (Rainford et al., 1964) have thought that the impairment results directly from one pattern of structural change that can be produced by either thiol-unrelated reagents or by thiol reagents. In the latter case we have to postulate that placing "foreign" groups at certain cysteyl-S locations produces structural disruptions as far away from these locations as the ATPase site.

On this simple picture mere bulk of the group attached could be important, and it is probably significant that those who have found some surviving function after extensive reaction used attaching groups of small bulk. From comparisons with space-filling models, we estimate that the group (–CN) attached by Wiedner et al. (1978) had 69% of the volume of the group ($-SCH_3$) attached by us and only 31% of the group ($-CH_2CH_2COOCH_3$) attached by Gaetjens et al. (1964). Thus, the cyano group is the smallest. However, Wiedner et al. (1978) found a secondary reaction in which HCN is displaced and a disulfide bond forms; perhaps it is the bulk difference or the propensity to secondary reaction of –CN that led them to results that are quantitatively very different from Gaetjens et al. or ours.

If placing groups at thiol locations produces structural disruptions, can we detect these disruptions and can we show that the disruptions go hand in hand with the placing of the group? A strong indication of a structural disruption is the precipitous decrease in ATPase observed when ATPase is plotted as a function of incubation time (Figure 2), i.e., when between 2 and 4 mol of MMTS per mol of S-1 become bound. Previously, we had thought that the corresponding precipitous increase in Ans binding independently confirms this surmise [cf. Duke et al. (1966)]. However, a careful simultaneous study of the kinetics of ATPase decrease and of bound Ans fluorescence increase (Figure 6A) has shown us that the variables are not synchronous; especially, a phase plot (Figure 6B) has shown that the relationship between processes is somewhat indirect. What has proved to be a better indicator is the information reported by a probe, 1,5-IAEDANS, at SH_1. This might be expected, since exploratory energy-transfer work (Haugland, 1975) has shown that SH_1 is near to the ATPase site (to which the structural disruptions are presumed to be communicated). The probe reports two kinds of information, the rotational correlation time, Φ, and the excited-state lifetime, τ. Decreases in τ are usually taken to indicate that the aqueous solvent has increased access to the probe (i.e., ability to lose energy through dielectric relaxation improves); thus, a decrement in τ can reflect a *local* change in the probe neighborhood. An increase Φ indicates either an increase in asymmetry or an increase in volume of the particle as a whole; thus, an increment in Φ indicates a *global* change. Mere placement of the blocking groups at thiol locations causes little

change in either τ or Φ [cf. Mendelson et al. (1975)], but, in the presence of ATP, τ is significantly smaller and Φ is significantly larger than in unblocked S-1. Therefore, it seems proper to say that in both the local and global sense, ATP addition produces a different structure in blocked than in unblocked S-1. Moreover, Figure 7 shows that these changes go hand in hand with the ATPase and actin affinity changes that blocking also produces. Note in passing that the foregoing interpretation is not subject to two frequent pitfalls encountered in probe work; often structural disruption increases mobility of the probe relative to the macromolecule to which it attaches; this is not the predominant effect of thiol blocking, however, because Φ increases rather than decreases. Also, thiol blocking is not simply diminishing the ability of ATP to bind to the ATPase site because the changes in Φ and τ are evident only after ATP addition.

In summary, the ATPase activity and the actin-binding affinity of S-1 do not appear to be processes in which thiol groups *as such* participate (because these properties survive even after all accessible thiols are blocked). Placing even rather small groups at certain thiol locations, however, indirectly impairs S-1 properties. The mechanism of this impairment seems to be that the thiol blocks produce structural disruption which in turn is transmitted to the ATPase and actin-binding sites. The disruption is adequately sensed by a 1,5-IAEDANS probe attached to thiol SH_1.

References

Bárány, M., & Bárány, K. (1959) *Biochim. Biophys. Acta 35*, 293.

Beinfeld, M. C., & Martonosi, A. N. (1975) *J. Biol. Chem. 250*, 7971.

Botts, J., Stone, D. B., Wang, A., & Mendelson, R. A. (1975) *J. Supramol. Struct. 3*, 141.

Boyer, P. D. (1954) *J. Am. Chem. Soc. 76*, 4331.

Duke, J. A., McKay, R., & Botts, J. (1966) *Biochim. Biophys. Acta 126*, 600.

Elzinga, M., Collins, J. H., Kuehl, W. M., & Adelstein, R. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2687.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375.

Gaetjens, E., Therattil-Antony, T., & Bárány, M. (1964) *Biochim. Biophys. Acta 96*, 554.

Hartshorne, D., & Morales, M. F. (1965) *Biochemistry 4*, 18.

Haugland, R. P. (1975) *J. Supramol. Struct. 3*, 338.

Highsmith, S. (1976) *J. Biol. Chem. 251*, 6170.

Highsmith, S., Mendelson, R. A., & Morales, M. F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 133.

Houk, W., & Ue, K. (1974) *Anal. Biochem. 62*, 66.

Kretzschmar, M., Mendelson, R. A., & Morales, M. F. (1978) *Biochemistry 17*, 2314.

Lim, S. T., & Botts, J. (1967) *Arch. Biochem. Biophys. 122*, 125.

Lin, T.-I., & Morales, M. F. (1977) *Anal. Biochem. 77*, 10.

Lowey, S., Slayter, H. S., Weeds, A. G., & Baker, H. (1969) *J. Mol. Biol. 42*, 1.

Mendelson, R. A., Morales, M. F., & Botts, J. (1973) *Biochemistry 12*, 2250.

Mendelson, R. A., Putnam, S., & Morales, M. F. (1975) *J. Supramol. Struct. 3*, 162.

Rainford, P., Hotta, K., & Morales, M. F. (1964) *Biochemistry 3*, 1218.

Sekine, T., & Kielley, W. W. (1964) *Biochim. Biophys. Acta 81*, 336.

Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766.

Spudich, J., & Watt, S. (1971) *J. Biol. Chem. 246*, 4366.

Takashi, R., Duke, J., Ue, K., & Morales, M. F. (1976) *Arch. Biochem. Biophys. 175*, 279.

Tonomura, Y., Tokura, S., & Sekiya, K. (1961) *Arch. Biochem. Biophys. 95*, 229.

Tonomura, Y., Appel, P., & Morales, M. F. (1966) *Biochemistry 5*, 515.

Weber, G., & Young, L. B. (1964) *J. Biol. Chem. 239*, 1415.

Westhaus, P. A., & Pohl, H. A. (1978) *J. Theor. Biol. 70*, 157.

Wiedner, H., Wetzel, R., & Eckstein, F. (1978) *J. Biol. Chem. 253*, 2763.

Young, D. M., Himmelfarb, S., & Harrington, W. F. (1965) *J. Biol. Chem. 240*, 2428.