Larsson, U. K., & Anderson, B. (1985) *Biochim. Biophys. Acta 809*, 396–402.
Ljungberg, U., Åkerlund, H.-E., & Andersson, B. (1984) *FEBS Lett. 175*, 255–258.
Lysko, K. A., Carlson, R., Taverna, R., Snow, J., & Brandts, J. F. (1981) *Biochemistry 20*, 5570–5576.
Manly, S. P., Matthews, K. S., & Sturtevant, J. M. (1985) *Biochemistry 24*, 3842–3846.
Melis, A., & Homann, P. H. (1978) *Arch. Biochem. Biophys. 190*, 523–530.
Miyao, M., & Murata, N. (1984) *FEBS Lett. 170*, 350–354.
Miyao, M., & Murata, N. (1985) *FEBS Lett. 180*, 303–308.
Mullet, J. E., & Arntzen, C. J. (1980) *Biochim. Biophys. Acta 589*, 100–117.
Nash, D., Miyao, M., & Murata, N. (1985) *Biochim. Biophys. Acta 807*, 127–133.
Petrouleas, V., & Diner, B. A. (1986) *Biochim. Biophys. Acta 849*, 264–275.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.
Privalov, P. L. (1980) *Pure Appl. Chem. 52*, 479–497.
Sandusky, P. O., & Yocum, C. F. (1984) *Biochim. Biophys. Acta 766*, 603–611.
Sandusky, P. O., Selvius DeRoo, C. L., Hicks, D. B., Yocum, C. F., Ghanotakis, D. F., & Babcock, G. T. (1983) in *The Oxygen Evolving System of Photosynthesis* (Inoue, Y., et al., Eds.) p 189, Academic Press, Tokyo.
Satoh, K. (1983) in *The Oxygen Evolving System of Photosynthesis* (Inoue, Y., et al., Eds.) p 27, Academic Press, Tokyo.
Sturtevant, J. M. (1974) *Annu. Rev. Biophys. Bioeng. 3*, 35–51.
Sundby, C., & Andersson, B. (1985) *FEBS Lett. 191*, 24–28.
Tang, X.-S., & Satoh, K. (1985) *FEBS Lett. 179*, 60–64.
Wensink, J., Dekker, J. P., & Van Gorkom, H. J. (1984) *Biochim. Biophys. Acta 765*, 147–155.
Zuber, H. (1985) *Photochem. Photobiol. 42*, 821–844.

Location of the Sites of Reaction of *N*-Ethylmaleimide in Papain and Chymotryptic Fragments of the Gizzard Myosin Heavy Chain†

Narindar Nath,‡ Sumitra Nag, and John C. Seidel*

Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114, and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

Received February 11, 1986; Revised Manuscript Received June 10, 1986

ABSTRACT: The thiol of the gizzard myosin heavy chain, which reacts most rapidly with *N*-ethylmaleimide (MalNEt), has been located in the subfragment 2 region of myosin rod by fragmentation of [^{14}C]-MalNEt-labeled myosin with papain and chymotrypsin. MalNEt reacts more slowly with thiols present in the 70- and 25-kilodalton (kDa) papain fragments of subfragment 1. The reaction of MalNEt with thiols present in these regions is increased on addition of ATP by factors of 2 and 10, respectively, when myosin is modified in 0.45 M NaCl where it is present in the extended, 6S conformation. The rate of increase of Mg^{2+}-activated adenosinetriphosphatase (ATPase) activity, which reflects the loss of ability of myosin to assume the folded, 10S conformation, and the rate of loss of K^+-EDTA-activated activity produced by MalNEt are both accelerated 5- to 10-fold on addition of ATP. The rates at which ATPase activities change agree closely to the reaction rates of MalNEt with the 25-kDa region of subfragment 1; therefore, the changes in these activities can be attributed to modification of a thiol of the 25-kDa segment. An increase in actin-activated ATPase activity produced by reaction of myosin with MalNEt in 0.45 M NaCl is accelerated by ATP by a factor of at least 4. Reaction with [^{14}C]MalNEt in the presence of MgATP and 0.2 M NaCl, where myosin is in the 10S form, inhibits the incorporation of radioactive MalNEt into the 25-kDa papain fragment of subfragment 1. It also prevents the increase in actin-activated ATPase activity and preserves the ability of myosin to assume the 10S form.

In reaching our present state of knowledge of the molecular basis of force generation, spectroscopic probes attached to the contractile proteins have played an important role, permitting investigation of structural changes and dynamic behavior of defined regions of these proteins (Morales et al., 1982; Gergely & Seidel, 1983). Cysteine residues, because of their chemical reactivity, have allowed attachment of probes at specific sites that can be defined in terms of their position in the amino acid sequence and eventually in the three-dimensional structure. The use of these sites has allowed estimates of intra- and intermolecular distances between sites on myosin and actin by spectroscopic (Burley et al., 1972; Marsh & Lowey, 1980; Takeshi et al., 1982) and chemical means (Reisler et al., 1974; Wells & Yount, 1979; Tao & Lamkin, 1981), leading to the proposal of a three-dimensional model of various reactive sites of the actin–S1 complex (Botts et al., 1984; dos Remedios & Cooke, 1984). The future usefulness of this approach will depend in part on the availability of additional sites for attachment of probes within the myosin and actin molecules.

Skeletal muscle myosin contains reactive thiols, SH-1 and SH-2 of the heavy chain (Kielley & Bradley, 1956), present in a 22-kDa tryptic peptide constituting the C-terminal region

†Supported by Grant HL-15391 from the National Institutes of Health and by a grant from the Muscular Dystrophy Association.
*Address correspondence to this author at Boston Biomedical Research Institute.
‡Present address: Department of Biochemistry, Punjab University, Chandigarh, India.

0006-2960/86/0425-6169$01.50/0

of S1[1] (Balint et al., 1978), 10 amino acids apart in the primary structure (Elzinga & Collins, 1977) and sufficiently close to each other in the tertiary structure to be cross-linked with bifunctional thiol reagents (Reisler et al., 1974; Wells & Yount, 1979; Wells et al., 1980). SH-1 is the most reactive thiol, with SH-2 becoming highly reactive in the presence of ATP or ADP (Sekine & Yamaguchi, 1963). Reaction of SH-1 with MalNEt abolishes K^+-EDTA-activated adenosinetriphosphatase (ATPase) activity and increases Ca^{2+}-activated activity, while subsequent reaction of SH-2 leads to a loss of all activity (Kielley & Bradley, 1956).

Gizzard myosin contains a reactive thiol in the 17-kDa light chain (Okamoto & Sekine, 1980; Bailin & Lopez, 1981; Chandra et al., 1985; Onishi, 1985) and at least three such groups in the heavy chain (Chandra et al., 1985; Onishi, 1985). The pattern of incorporation of MalNEt into gizzard myosin indicates the presence of rapidly and slowly modified thiols, the former including thiols in the myosin rod and the 17-kDa light chain and the latter including at least two thiols located in the S1 heavy chain (Chandra et al., 1985; Onishi, 1985).

The thiols of smooth muscle myosin are also of interest because thiol-directed reagents enhance actin-activated ATPase activity as does enzymatic phosphorylation of the 20-kDa light chain (Seidel, 1979). These reagents inhibit formation of the 10S conformation (Chandra et al., 1985) and stabilize myosin filaments (Chandra et al., 1985; Onishi, 1985), effects that are also produced by light-chain phosphorylation (Suzuki et al., 1978, 1982). Molecular changes associated with the 6S–10S transition may play a role in regulation of actin-activated ATPase activity by phosphorylation (Suzuki et al., 1978, 1982, 1985; Ikebe & Hartshorne, 1984, 1985).

The present studies show that the most reactive thiol of the myosin rod is in the S2 region. The thiol, whose reaction with MalNEt inhibits K^+-EDTA-activated ATPase activity and increases Mg^{2+}-activated activity with and without actin, is in the C-terminal 25-kDa region of S1, the region analogous to that containing the SH-1 and SH-2 thiols of skeletal muscle myosin (Balint et al., 1978). Reaction of this C-terminal thiol with MalNEt also increases the Mg^{2+}-activated and actin-activated ATPase activities.

Materials and Methods

The proteins used in these studies were prepared by standard procedures used in previous work (Chandra et al., 1985). Digestion of myosin with papain was carried out in a solution containing 0.5 M NaCl, 10 mM MOPS, pH 7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10–15 mg/mL myosin, and 5 μg/mL papain, the reaction being terminated with iodoacetic acid at a final concentration of 1–5 mM. Papain (1 mg/mL) was activated for 1 h at 37 °C in a solution containing 50 mM freshly dissolved cysteine, 10 mM EDTA, and 10 mM Tris, pH 8.0. Subfragment 1 was obtained from the supernatant fraction of a 16-min digest of myosin after dialysis against low ionic strength buffer, a solution containing 35 mM NaCl, 10 mM MOPS, pH 7.0, 10 mM $MgCl_2$, 0.05 mM EGTA, and 0.1 mM dithiothreitol, by sedimentation at 18 000*g* for 30 min to remove insoluble myosin fragments. Myosin rod was obtained from the insoluble fraction of a 40-min papain digestion of myosin followed by dialysis against the low ionic strength buffer described above without dithiothreitol. The pellet obtained after centrifugation at 18 000*g* for 30 min was redissolved in a solution containing 0.5 M NaCl, 10 mM MOPS, pH 7.0, 0.1 mM EDTA, and 0.1 mM dithiothreitol followed by an additional cycle of precipitation and resolution when required. Chymotryptic digestion of gizzard myosin was carried out as described elsewhere (Weeds & Pope, 1977; Seidel, 1980).

Polyacrylamide gel electrophoresis was carried out in sodium dodecyl sulfate on 12% slab gels with 6% stacking gels, using the discontinuous Tris–glycine buffer system (Laemmli, 1970), or on 5-mm, 8% or 10% disk gels (Weber & Osborn, 1969). The acrylamide/bis(acrylamide) ratio was 36.5 in all cases.

Myosin reacted with MalNEt for the indicated times at 0 °C in a solution containing 0.45 M NaCl, 40 mM MOPS, pH 7.5, and 8–12 mg/mL myosin. Unphosphorylated myosin was used unless otherwise noted. The reaction was initiated by addition of MalNEt to a final concentration of 0.3–1.0 mM, terminated by addition of dithiothreitol to a final concentration of 10 mM, and the ATPase activities were determined.

MalNEt labeled with ^{14}C at positions 2 and 3 of the maleimide ring—specific activity 40–45 mCi/mmol—was obtained from New England Nuclear or Amersham Corp. The radioactive MalNEt was diluted 10 times with carrier MalNEt of the desired concentration and the reaction with myosin was carried out in a solution containing 0.45 M NaCl, 40 mM MOPS, pH 7.5, 0.1 mM dithiothreitol, and 8–12 mg/mL myosin as described above. Radioactivity bound to the heavy and light chains and proteolytic fragments of [^{14}C]MalNEt-labeled myosin was determined by separation of stained bands of the sodium dodecyl sulfate gels, addition of 200 μL of 30% H_2O_2, and incubation overnight at 40 °C. If necessary, more H_2O_2 was added and the digestion was continued until gel slices were completely dissolved. Radioactivity was determined by liquid-scintillation counting.

To prevent further digestion by papain during electrophoresis, papain digests were heated for 2 min in boiling water immediately after the addition of the detergent. Autoradiography was carried out with polyacrylamide gels, stained with Coomassie Blue, vacuum dried, and exposed to X-Omal-XAR5 film for 1–2 weeks.

Mg^{2+}-activated ATPase activity was measured in solutions or suspensions containing 150 mM NaCl, 20 mM MOPS, pH 7.5, 1 mM ATP, 0.1 mM EGTA, 1 mM $MgCl_2$ and 1.0 mg/mL myosin. K^+-EDTA-activated ATPase activity was measured in solutions containing 0.6 M KCl, 40 mM MOPS, pH 7.5, 5 mM EDTA, 5 mM ATP, and 0.5 mg/mL myosin. Incubations were carried out at 25 °C and terminated with an equal volume of 10% trichloroacetic acid, and the concentrations of inorganic phosphate were determined (Fiske & SubbaRow, 1925).

Sedimentation studies were carried out in a Beckman Model E analytical ultracentrifuge (Chandra et al., 1985).

Results

Location of a Rapidly Reacting Thiol, SH-A, in Subfragment 2. To locate the site of the heavy chain that reacts most raipdly with MalNEt, myosin was labeled for 5 min with [^{14}C]MalNEt, and the large peptide fragments obtained on papain digestion (see Figure 1) were separated by gel electrophoresis. Autoradiographs (Figure 2A,F) indicate that most of the radioactivity released from the heavy chain during papain digestion appears initially in the 150- and 125-kDa papain peptides that constitute the myosin rod. As the digestion proceeds, the radioactivity of the 150-kDa fragment appears in the 125-kDa polypeptide chain; upon solubilizing the gel bands at least 75% of the radioactivity originally present

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; HMM, heavy meromyosin; LMM, light meromyosin; MalNEt, *N*-ethylmaleimide; MOPS, 4-morpholinepropanesulfonic acid; S1, subfragment 1; S2, subfragment 2.

Table I: Distribution of Radioactivity (cpm) of [^{14}C]MalNEt-Labeled Myosin in Papain Fragments of Heavy Chain[a]

	digestion time (min)								
	rapid phase				slow phase				
$M_r \times 10^{-3}$	0	8	16	40	0	8	16	32	50
200	723	473	338	144	1551	769	430	130	41
145		125	157	249		132	110	60	17
120		161	400	354		26	22	24	23
95		56	74	92		211	323	448	360
70		31	46	71		133	165	307	372
25			58	59			62	115	140

[a] Myosin was incubated with 0.8 mM [^{14}C]MalNEt, specific activity 6450 cpm/nmol, for 5 min (rapid phase) or with cold MalNEt for 5 min followed by 55 min with [^{14}C]MalNEt, specific activity 10640 cpm/nmol (slow phase). The ^{14}C-labeled myosin was digested with papain, 5 μg/mL, as described under Materials and Methods for the times indicated. Gel electrophoresis was carried out in sodium dodecyl sulfate, and the radioactivity in each polypeptide chain was determined as described under Materials and Methods.

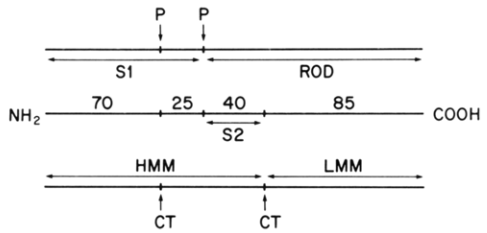

FIGURE 1: Schematic diagram of cleavage sites of the heavy chain of gizzard myosin by papain and chymotrypsin. The 70- and 25-kDa fragments constitute the heavy chain of S1 (Sobieszek & Small, 1976), the 72- and 62-kDa fragments make up the heavy chain of HMM (Seidel, 1978, 1980; Okamoto et al., 1980), and the 40-kDa peptide constitutes S2 (Cross et al., 1984; Suzuki et al., 1984).

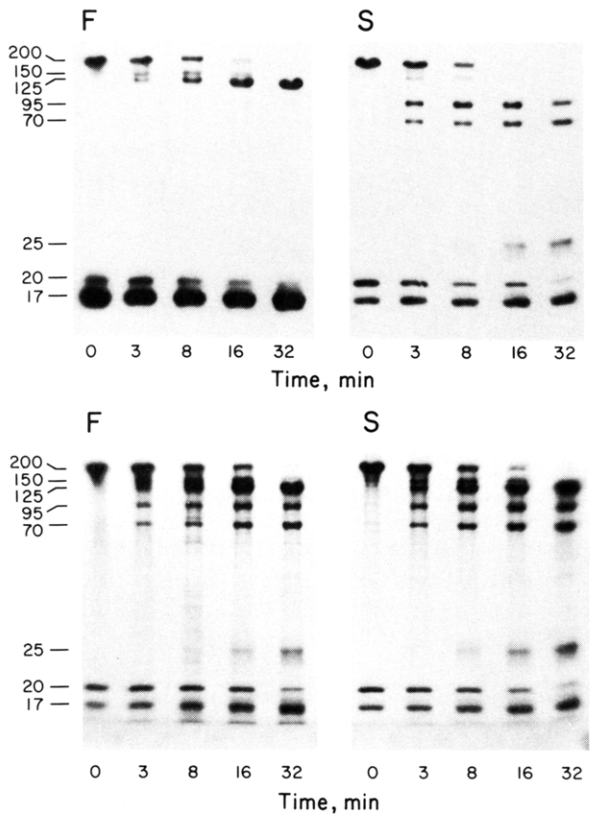

FIGURE 2: Distribution of MalNEt blocked sites in papain fragments of the heavy chain during the course of papain digestion. To block the rapidly reacting sites, 8–10 mg/mL myosin was incubated with 0.8 mM ^{14}C-labeled MalNEt for 5 min (F). To block the slowly reacting sites, myosin was incubated with 0.8 mM MalNEt for 5 min followed by addition of essentially carrier-free [^{14}C]MalNEt and further incubation for 55 min (S). The labeled myosin was digested with papain for the times indicated, gel electrophoresis was carried out in sodium dodecyl sulfate, and the radioactivity in each polypeptide chain was determined by autoradiography as described under Materials and Methods. (A) Autoradiogram; (B) Coomassie blue stained gel.

Table II: Distribution of Radioactivity in Chymotryptic Peptides of [^{14}C]MalNEt-Labeled Myosin[a]

	HMM		LMM and undigested myosin	
$M_r \times 10^{-3}$	bound radioactivity (cpm)	bound MalNEt (nmol/mg)	bound radioactivity (cpm)	bound MalNEt (nmol/mg)
200			164	0.87
135	707	2.45	145	0.77
85			7	<0.10
72	334	1.16	48	0.25
65	416	1.44	12	<0.10

[a] Myosin was labeled with 0.8 mM ^{14}C-labeled MalNEt for 25 min, with 10.5 mol of MalNEt per mole of myosin being incorporated, and digested with 0.1 mg/mL chymotrypsin for 10 min. Digests were separated into soluble and insoluble fractions after dialysis against a solution containing 35 mM NaCl, 10 mM MOPS, pH 7.0, 10 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM dithiothreitol. The results represent the average of two experiments in which 36% and 53% of the original radioactivity bound to the MalNEt-labeled myosin was recovered in the three bands of HMM and the five bands of LMM and undigested myosin of the stained gel. The amount of bound label represents the number of nmoles of MalNEt recovered from each Coomassie blue stained band divided by the number of milligrams of digested myosin applied to the gel. The number of nmoles of MalNEt was obtained by dividing the cpm in each band by the specific activity of [^{14}C]MalNEt of 11880 cpm/nmol of MalNEt.

in the heavy chain is found in this peptide (Table I). Radioactivity in the 20- and 17-kDa light chains is not included in this calculation. No radioactivity is detected in heavy-chain fragments having molecular weights less than 125 kDa by autoradiography. A small amount of radioactivity, not more than 15% of the total, was detected in the 95-, 70-, and 25-kDa fragments of S1, which can be attributed to the reaction of slowly modified sites that become partially labeled during the rapid phase of the reaction.

Chymotryptic fragmentation of myosin labeled with [^{14}C]MalNEt is accompanied by the appearance of almost all of the radioactivity in HMM—the 135-, 72-, and 65-kDa fragments—with less than 2% in LMM, the 85-kDa fragment (Table II). This result, together with the observation that radioactivity is present in the rod but not in LMM indicates that SH-A is part of the S2 region. The location of this thiol in S2 is confirmed directly by chymotryptic fragmentation of [^{14}C]MalNEt-labeled myosin rod, which results in release of a soluble 40-kDa fragment of S2 containing at least 80% of the radioactivity (Figure 3). In contrast, the 85-kDa fragment (LMM) contains less than 2% with no other peptide fragments being observed.

Location of the More Slowly Reacting Thiols in Subfragment 1. Papain digestion of myosin in which the more reactive thiols were blocked for 5 min with nonradioactive MalNEt, followed by addition of essentially carrier free [^{14}C]MalNEt

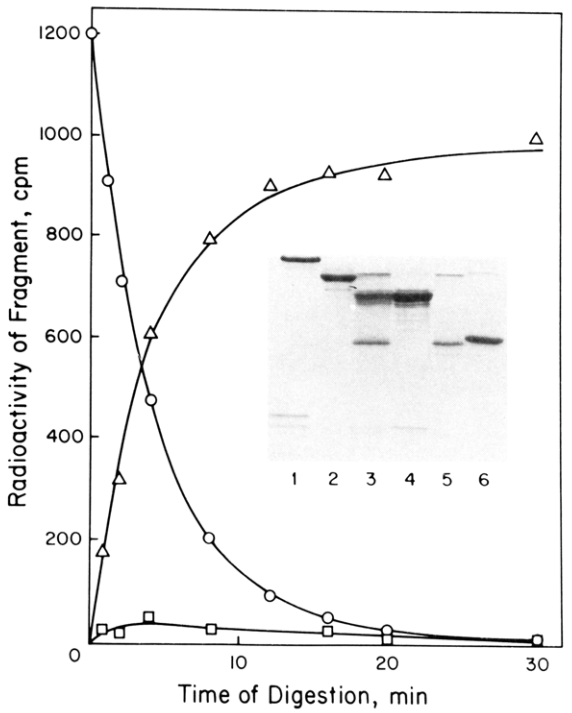

FIGURE 3: Distribution of radioactivity of bound MalNEt in chymotryptic fragments of gizzard myosin rod. The rapidly reacting sites of the heavy chains of gizzard myosin were labeled as described in legend to Figure 2 and the labeled myosin was digested with papain to prepare myosin rod as described under Materials and Methods. The ^{14}C-labeled rod was digested with chymotrypsin, 0.1 mg/mL, for times indicated, and digests were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Bands corresponding to undigested rod, LMM and S2, were digested with hydrogen peroxide, and radioactivity, expressed in cpm per gel bands, was determined as described under Materials and Methods: (○) rod; (△) S-2; (□) LMM. The inset is a Coomassie blue stained electrophoretic gel of the products of chymotryptic digestion of the myosin rod. The digestion and separation of S2 and LMM were carried out as described under Materials and Methods: (1) gizzard myosin, heavy chain 200 kDa; (2) gizzard myosin rod, 125 kDa (Cross et al., 1984); (3) unfractionated chymotryptic digest of myosin rod; (4) light meromyosin, 85 kDa (Okamoto et al., 1980; Suzuki et al., 1984); (5) subfragment 2, 40 kDa (Cross et al., 1984; Suzuki et al., 1984); (6) skeletal muscle actin, 42 kDa.

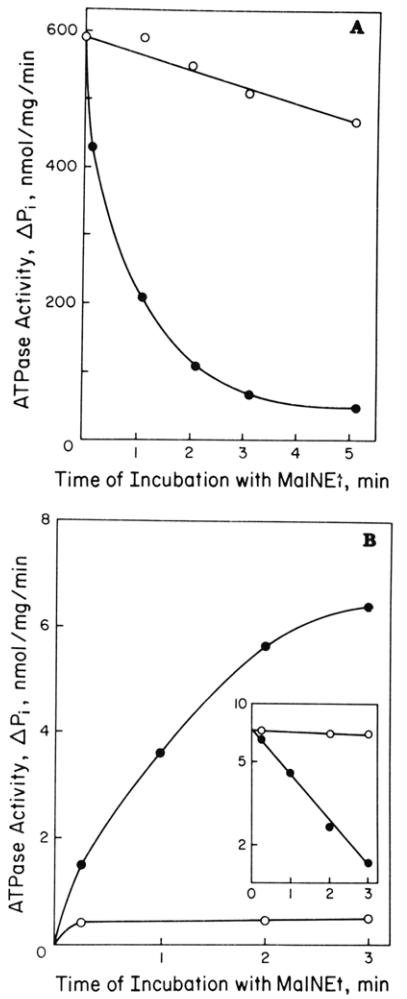

FIGURE 4: Effect of ATP on (A) the loss of K^+-EDTA-activated ATPase activity and on (B) the increase in Mg^{2+}-activated ATPase activity. Myosin was incubated for the times indicated in a solution containing 0.45 M NaCl, 40 mM MOPS, pH 7.0, 0.1 mM EDTA, and 0.5 mM MalNEt with (●) or without (○) 1 mM ATP. The reaction was terminated by addition of dithiothreitol to a final concentration of 10 mM and ATPase activities determined as described under Materials and Methods with 0.6 M KCl and 5 mM EDTA (A) or 1 mM $MgCl_2$ (B).

and further incubation for 55 min, was employed to locate the slowly reacting sites. During the initial stages of papain digestion essentially all of the radioactivity released from the heavy chain is found in the 150-, 95-, and 70-kDa fragments that contain all or part of S1 (Figure 2A,S). With longer digestion times the 95- and 150-kDa fragments are digested and some of their radioactivity appears in the 25-kDa fragment. Several peptides of 22–24 kDa are formed from the 25-kDa fragment on longer digestion, and radioactivity lost from the 25-kDa region appears in these smaller fragments; therefore their radioactivity is included when determining that of the 25-kDa fragment. Throughout the digestion the 125-kDa rod peptide never had more than 2–3% of the total radioactivity as estimated by scintillation counting of sliced gels (Table I). The radioactivity in the 95-kDa fragment appears with time in the 70- and 25-kDa fragments.

Effects of Blocking of Thiols on ATPase Activity. Because ATP increases the reactivity of the SH-2 thiols of skeletal muscle myosin (Sekine & Yamaguchi, 1963), we investigated the effect of ATP on the loss of K^+-EDTA-activated ATPase activity induced by MalNEt (Figure 4A). Activity is lost slowly without ATP but the rate of change is greatly accelerated by ATP. This effect of ATP is observed in the presence or absence of Mg^{2+}. In the absence of ATP, MalNEt produces a small increase in the Mg^{2+}-activated activity, but again the addition of ATP greatly accelerates the rate (Figure 4B). To obtain rate constants for the changes in Mg^{2+}-activated activity the experimental data were plotted according to the equation $\log (V_m - V_t) = -kt + c$, where V_t is the activity at time t, V_m is the activity when the reaction with MalNEt is complete, and k is a pseudo-first-order rate constant. Because the Mg^{2+}-activated ATPase activity begins to decrease on prolonged incubation with MalNEt, V_m could not be obtained with certainty by prolonging the reaction; therefore the data were plotted with different values of V_m between 5 and 10 nmol mg^{-1} min^{-1} and a value of 7.5 provided the closest approximation to a straight line. Constants determined for reaction with MalNEt in the presence and in the absence of ATP differ by a factor of about 10 (Table III).

Comparison of the rates of MalNEt-induced changes in ATPase activity with the rate of incorporation of [^{14}C]MalNEt into the heavy and light chains and their proteolytic fragments was used to locate the sites involved in changes in activity. ATP accelerates the rate of incorporation of MalNEt into the heavy chain but does not influence incorporation into either light chain (Figure 5). ATP accelerates the incorporation [^{14}C]MalNEt into both the 25- and 70-kDa fragments of S1

Table III: Rate Constants for Incorporation of MalNEt into S1 Peptides and for MalNEt-Induced Changes in ATPase Activity[a]

	rate constant (min^{-1})	
	-ATP	+ATP
K^+-EDTA-activated ATPase	0.04	0.39
Mg^{2+}-activated ATPase	0.01	0.53
incorporation into 25-kDa peptide	0.03	0.56
incorporation into 70-kDa peptide	0.05	0.14

[a] Myosin (7–9 mg/mL) was incubated in solutions containing 0.45 M NaCl, 40 mM MOPS, pH 7.5, 0.1 mM EDTA, and 0.5 mM MalNEt with and without 1 mM ATP, and rates of incorporation and rates of changes in ATPase activity were determined from data shown in Figure 5.

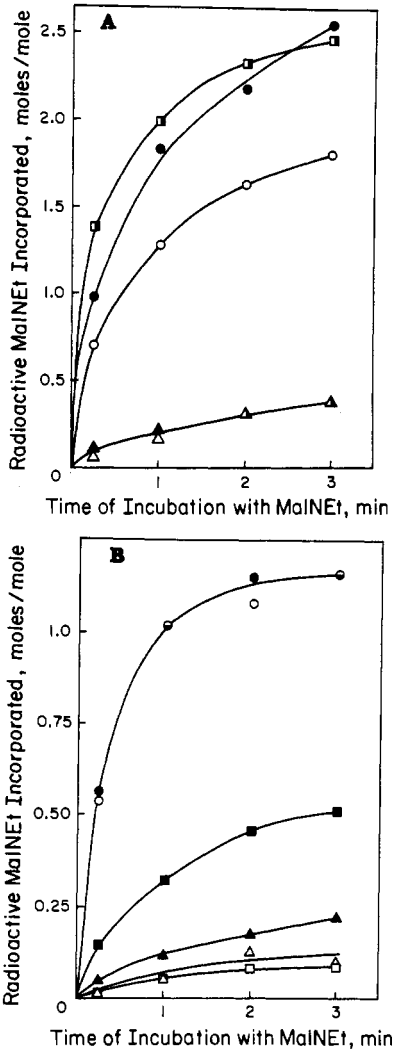

FIGURE 5: Effect of ATP on incorporation of [^{14}C]MalNEt into myosin subunits and their papain fragments. Myosin was labeled as described in the legend to Figure 4, and the radioactivity in each subunit and proteolytic fragment was determined as described under Materials and Methods: (A) (○, ●) heavy chain, (△, ▲) 20-kDa light chain, (□, ■) 17-kDa light chain; (B) (○, ●) 125-kDa rod, (△, ▲) 70-kDa fragment, (□, ■) 25-kDa fragment. Open symbols, reaction with MalNEt in absence of ATP; closed symbols, reaction in the presence of 1 mM ATP.

but does not affect incorporation into and myosin rod, ruling out the light chains and rod as the location of the thiol group responsible for the changes. ATP produces a 10-fold enhancement of incorporation of MalNEt into the 25-kDa fragment but only a 2- to 3-fold enhancement of incorporation into the 70-kDa fragment. Rate constants estimated for the increase in Mg^{2+}-activated ATPase activity agree more closely

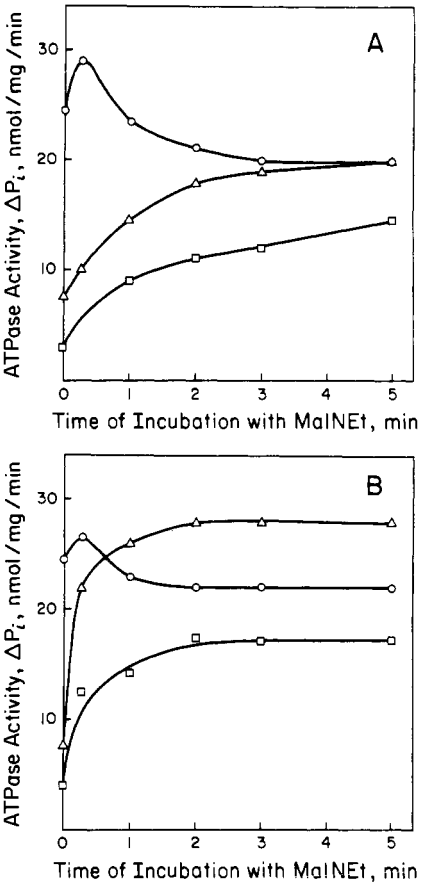

FIGURE 6: Changes in actin-activated ATPase activity of myosin induced by reaction with MalNEt at 0.45 M NaCl in the absence (A) and presence (B) of ATP. Unphosphorylated myosin was modified with 0.5 mM MalNEt for 1 min in the presence or absence of 1 mM ATP, as described under Materials and Methods. The activity in the presence of actin was measured with myosin light-chain kinase and with 0.2 mM EGTA or 0.2 mM $CaCl_2$ to obtain the activities of unphosphorylated and phosphorylated myosins, respectively. There was no difference between activity of phosphorylated myosin whether myosin was phosphorylated before or after reaction with MalNEt (Chandra et al., 1985). The ATPase assay medium contained 60 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, and 1.0 mg/mL myosin: (○) 0.5 mg/mL actin, 50 μg/mL myosin light chain kinase, and 0.2 mM $CaCl_2$; (△) 0.5 mg/mL actin, 50 μg/mL myosin light chain kinase, and 0.2 mM EGTA; (□) 0.2 mM EGTA.

with those obtained for the rate of incorporation of MalNEt into the 25-kDa fragment than they do with for the incorporation into the 70-kDa peptide (Table III). The same is true for changes in K^+-EDTA-activated ATPase activity. The incorporation of MalNEt into the 70-kDa fragment is too slow in the presence of ATP to account for changes in activity.

To determine whether reaction of MalNEt with SH-C—the reactive thiol in the 25-kDa papain fragment—increases the actin-activated ATPase activity, myosin was modified with MalNEt in the presence of 0.45 or 0.2 M NaCl, with and without ATP, and the activity was measured in the presence and absence of actin. In 0.45 M NaCl, incorporation of MalNEt into both SH-C and SH-D, the reactive thiols of the 25- and 70-kDa papain fragments, respectively, is accelerated by ATP (Figure 5), while in 0.2 M NaCl, MgATP induces a transition to the 10S form (Suzuki et al., 1978), accompanied by a decrease in the rate of incorporation of MalNEt into the 25-kDa fragment and an increase in the rate of incorporation into the 70-kDa fragment (Table IV). When the reaction with MalNEt is carried out at high ionic strengths, myosin is in the 6S form and Mg^{2+}-activated and actin-activated ATPase activities of unphosphorylated myosin increase at

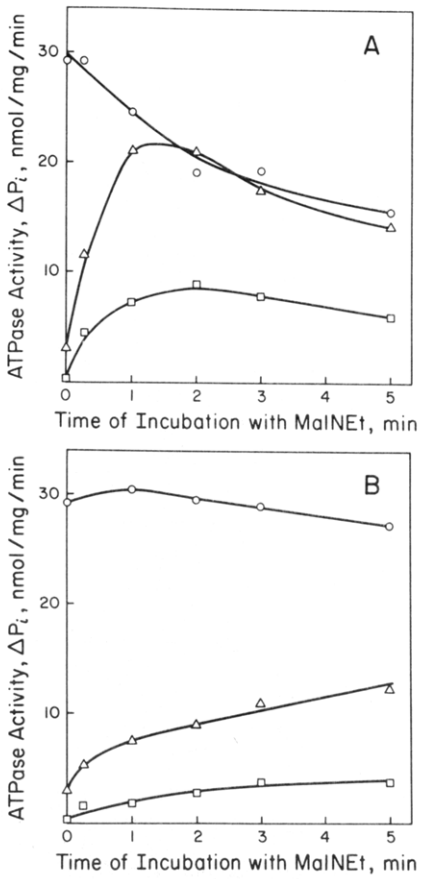

FIGURE 7: Changes in actin-activated ATPase activity of myosin induced by reaction with MalNEt at 0.2 M NaCl in the absence (A) and presence of MgATP (B). Myosin was modified with 0.5 mM MalNEt for the indicated times in a solution containing 0.2 M NaCl, 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 2 mM $MgCl_2$. The ATPase activities were measured as described in the legend to Figure 6. The symbols are all the same as described in Figure 6.

essentially the same rate (Figure 6). Without ATP the reaction is half complete in 1 min but with ATP the reaction is half complete within 15 s. The actin-activated activity of phosphorylated myosin decreases slightly on reaction with MalNEt either in the presence or in the absence of ATP.

In 0.2 M NaCl, myosin assumes the 10S form in the presence of MgATP with a concomitant decrease in the reactivity of SH-C (Onishi, 1985). Both SH-C and SH-D react with ^{14}C-labeled MalNEt at 0.2 M NaCl in the absence of ATP as indicated by the incorporation of radioactivity into both 70- and 25-kDa papain fragments of the myosin head, but in the presence of MgATP the reaction of SH-C is strongly inhibited (Table III). Without ATP, MalNEt increases the activity of unphosphorylated myosin for the first 2 min and then decreases it, in parallel with the decrease seen for phosphorylated myosin (Figure 7A). In the presence of MgATP, myosin assumes the 10S form and the ATPase activity undergoes little change (Figure 7B). These effects of MalNEt are all consistent with the blocking of SH-C increasing actin-activated ATPase activity.

To determine whether reaction of MalNEt with SH-C would inhibit formation of 10S myosin, reaction with MalNEt was carried out with and without MgATP and sedimentation velocity was measured. When myosin reacts with MalNEt at 0.45 M NaCl with ATP, where SH-C is reactive, only a single 6S peak is observed, indicating that myosin modified at SH-C does not assume the 10S form (Figure 8). In the absence of ATP, the sedimentation pattern shows the presence of two peaks sedimenting at 6S and 10S, the appearance of

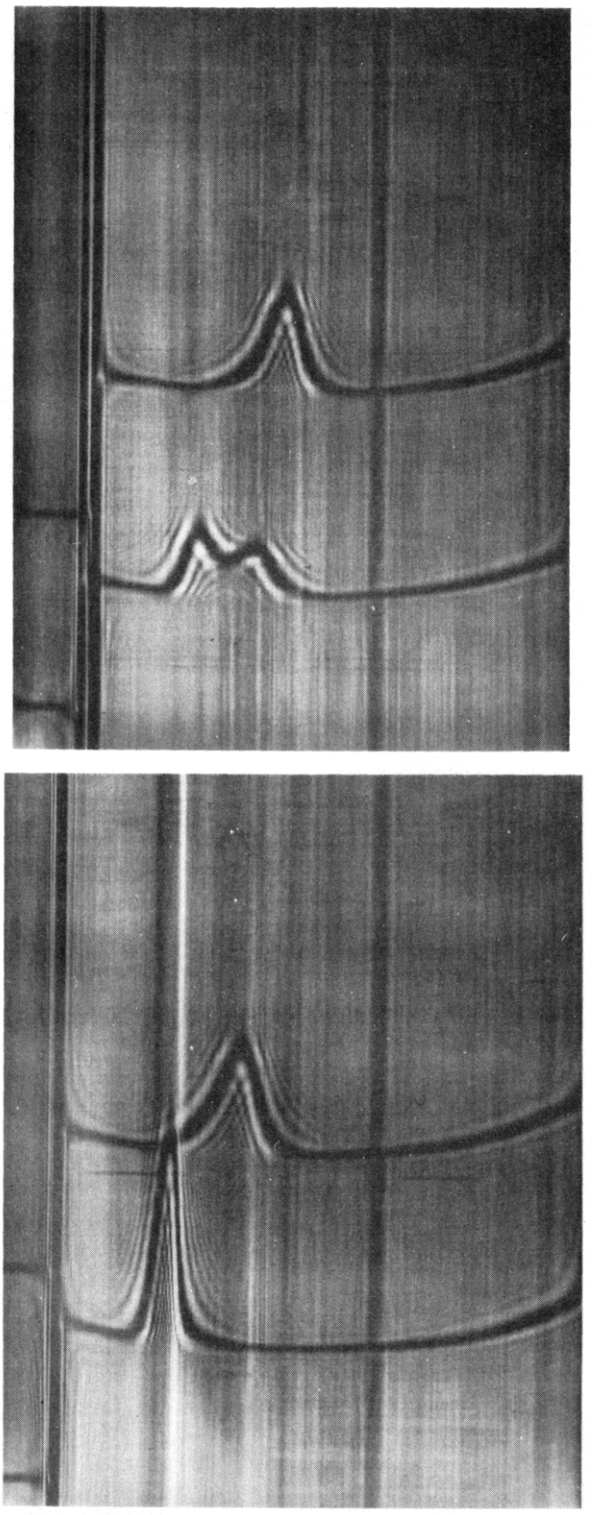

FIGURE 8: Sedimentation pattern of native myosin and myosin reacted with MalNEt in the presence of 0.45 M NaCl. Unphosphorylated myosin was incubated with 0.5 mM MalNEt at 0 °C for 1 min in a solution containing 0.45 M NaCl, 40 mM MOPS, pH 7.5, and 8–12 mg/mL myosin, as described under Materials and Methods. The reaction with MalNEt was carried out in the absence (A) and the presence (B) of 1 mM ATP. Sedimentation was carried out at 40 000 rpm and 20 °C in a solution containing 0.2 M NaCl, 20 mM MOPS, pH 7.5, 1 mM ATP, 0.1 mM EGTA, 2 mM $MgCl_2$, and 1.0 mg/mL myosin. Bar angle 65°, standard cell, MalNEt-modified myosin; wedge cell, native myosin.

the 6S peak suggesting that blocking of other thiols may also produce similar effects. Untreated myosin under these conditions sediments as a single peak having a velocity of 10S (not shown). Reaction with MalNEt in 0.2 M NaCl results in the

Table IV: Incorporation of [^{14}C]MalNEt into the Subfragment 1 Region of Myosin in the 6S and 10S Conformations[a]

	radioactivity incorporated (cpm)		
		1 mM ATP, 1 mM $MgCl_2$	1 mM ATP
25-kDa peptide	151	41	385
70-kDa peptide	142	185	252

[a] Myosin (6.7 mg/mL) was incubated for 3 min in a solution containing 0.15 M NaCl, 10 mM MOPS, pH 7.5, 1 mM $MgCl_2$, and 0.5 mM [^{14}C]MalNEt, specific activity 20 150 cpm/nmol, with and without 1 mM ATP. The labeled myosin was digested with papain, the resulting peptides were separated by gel electrophoresis in the presence of sodium dodecyl sulfate, and the stained bands were digested for scintillation counting, as described under Materials and Methods.

appearance of a single 6S peak under conditions where the untreated myosin sediments at 10S (not shown). The appearance of a 6S peak, when the reaction with MalNEt occurs in the presence of MgATP where SH-C is unreactive, provides additional evidence that blocking of thiols other than SH-C may influence the transition to the 10S conformation.

Discussion

The location of SH-A in subfragment 2 places it within the first 326 amino acid residues of the gizzard rod (Suzuki et al., 1984). This region of the skeletal muscle myosin rod contains only two pairs of cysteine residues at positions 68 and 110 (Capony & Elzinga, 1981; Lu & Lehrer, 1984). Although SH-A may be at any position within the S2 region, if it were to correspond to one of the two cysteines of short S2, it would be in the N-terminal third of S2, not more than 15–20 nm from the S1–S2 junction based on a length of S2 of 45–50 nm (Cross et al., 1984; Suzuki et al., 1985). It is unlikely that SH-A is part of the hinge region, which in skeletal muscle myosin links HMM and LMM (Sutoh et al., 1978), since in gizzard myosin virtually all of this region is part of LMM (Okamoto et al., 1980; Cross et al., 1984; Suzuki et al., 1984). Although MalNEt reacts with sites in both the S2 region and the C-terminal segment of S1, IAEDANS appears not to react with sites in the S2 region (Onishi, 1985; Onishi & Watanabe, 1985), suggesting that the reactive nature of the thiol of S2 does not extend to all thiol blocking agents.

Reaction of gizzard myosin with IAEDANS (Onishi, 1985; Onishi & Watanabe, 1985) or MalNEt (Chandra et al., 1985) decreass the K^+-EDTA-activated ATPase activity, a result characteristic of the blocking of the SH-1 thiol of skeletal muscle myosin (Kielley & Bradley, 1956). The SH-1 thiol is in the C-terminal region of S1, which can be isolated as a 22-kDa tryptic fragment by gel electrophoresis in solutions containing sodium dodecyl sulfate (Balint et al., 1978). The cysteine residue of gizzard myosin, whose reaction with IAEDANS inhibits K^+-EDTA-activated ATPase activity, has been identified by Onishi et al.[2] as SH-1; the amino acid sequence around the reactive cysteine is identical with that containing the SH-1 thiol of skeletal muscle myosin (Yamashita et al., 1964; Elzinga & Collings, 1977). The reaction of the same cysteine residue of gizzard myosin with MalNEt appears to be responsible for the loss of K^+-EDTA-activated ATPase activity (Chandra et al., 1985) and the increase in Mg^{2+}-activated activity (Figure 5). Comparison of the present results with those obtained with IAEDANS (Onishi, 1985; Onishi & Watanabe, 1985) shows that both reagents react with the C-terminal region of S1. On digestion of gizzard myosin with papain, this region is released as a 25-kDa fragment, and on digestion with trypsin it is part of a 68-kDa fragment that also contains the subfragment 2 region (Onishi & Watanabe, 1985). Location of the MalNEt-blocked thiol in the 25-kDa papain fragment is based on the agreement between the rate of incorporation of MalNEt into this fragment and the rates of changes in ATPase activity.

The effects of ATP and MgATP on the reaction of MalNEt with myosin—as monitored by the actin-activated ATPase activity—provide evidence that blocking of SH-1 increases the enhancement of ATPase activity produced by actin. The changes in the actin-activated activity of phosphorylated myosin suggest that reaction of SH-1 with MalNEt decreases activity while reaction of the thiol of the 70-kDa fragment (SH-D) may enhance it. Similar results have been obtained with 1,5-IAEDANS (Onishi, 1985; Onishi & Watanabe, 1985).

The present results indicate that ATP can have opposite effects on the reactivity of SH-C, depending whether the reaction with MalNEt is carried out in 0.2 or 0.45 M NaCl. At 0.45 M NaCl, addition of ATP accelerates incorporation of MalNEt into the C-terminal segment of S1, but at 0.2 M NaCl it inhibits incorporation of MalNEt into this segment (Table IV). This difference can be attributed to the conformational state of myosin during the reaction with MalNEt. At 0.45 M NaCl, myosin is in the 6S form in the presence or absence of ATP, while at 0.2 M NaCl it assumes the 10S form on addition of ATP (Suzuki et al., 1978). Thus ATP accelerates the reaction with MalNEt when myosin remains in the 6S form, while the reaction of SH-1 is blocked on formation of the 10S state as shown earlier with IAEDANS (Onishi, 1985).

The more rapid increase in Mg^{2+}-activated ATPase activity seen in previous studies in response to MalNEt (Chandra et al., 1985) than found in the present work appears to be attributable to the use of higher Mg^{2+} concentrations, which stabilize myosin filaments and increase their activity (Suzuki et al., 1978). Because the reaction of gizzard myosin with MalNEt or IAEDANS increases the stability of myosin filaments (Chandra et al., 1985; Onishi, 1985; Onishi & Watanabe, 1985), the faster increase in activity induced by MalNEt at high Mg^{2+} concentrations may involve an increase in filament stability superimposed on an increase in the activity of myosin monomers (Chandra et al., 1985). Blocking of the reactive thiols of the 17-kDa light chain or those of the S2 region may play a role in stabilization of myosin filaments. The slower increase in activity seen in the present experiments at low Mg^{2+} concentrations and 0.15 M NaCl where myosin is monomeric apparently involves only an increase in activity of the monomers themselves, which appears to be attributable to a transition from the 10S to the 6S form (Chandra et al., 1985).

Changes in the Mg^{2+}-activated ATPase activity of monomeric myosin produced by MalNEt are associated with loss of ability of the modified myosin to assume the folded, 10S conformation (Chandra et al., 1985). The association of the blocking of SH-1 with changes in Mg^{2+}-activated ATPase activity implies that blocking of SH-1 is responsible for the shift toward the 6S form. The present results provide evidence supporting this view showing that the reaction of SH-1 with MalNEt is accompanied by a loss of the ability of myosin to assume the 10S conformation. The transition from the 6S to the 10S form involves two distinct structural changes: bending of the tail accompanied by apparent interaction with the neck region (Trybus et al., 1982; Suzuki et al., 1982; Onishi & Wakabashi, 1982) and reorientation of the heads, such that

[2] H. Onishi, T. Maita, T. Miyanishi, G. Matsuda, and S. Watanbe, personal communication.

they extend back toward the tail in the 10S form (Onishi & Wakabashi, 1982). HMM undergoes a similar transition involving two forms having sedimentation coefficients of 7.5 and 9S, whose Mg^{2+}-activated ATPase activities differ by a factor of 10 (Suzuki et al., 1985). The 7.5–9S transition is accompanied by changes in orientation of the heads relative to the tail but not by folding of the tail, suggesting that the changes in activity involve the junctional region of the molecule between S1 and S2. This raises the question of whether blocking of SH-1 by MalNEt may affect Mg^{2+}-activated ATPase activity via changes in the orientation of the heads that are in turn mediated through the S1–S2 junction.

ACKNOWLEDGMENTS

We thank Aida Carlos and Remy Nazareno for excellent technical assistance.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3; *N*-ethylmaleimide, 128-53-0.

REFERENCES

Bailin, G., & Lopez, F. (1981) *Biochim. Biophys. Acta 668*, 46–56.

Balint, M., Wolf, I., Tarcsafalvi, A., Gergely, J., & Sreter, F. A. (1978) *Arch. Biochem. Biophys. 190*, 793–799.

Botts, J., Takashi, P., Torgerson, T., Hozumi, T., Muhlrad, A., Mornet, D., & Morales, M. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2060–2064.

Burley, R. W., Seidel, J. C., & Gergely, J. (1972) *Arch. Biochem. Biophys. 150*, 792–796.

Capony, J., & Elzinga, M. (1981) *Biophys. J. 33*, 148a.

Chandra, T. S., Nath, N., Suzuki, H., & Seidel, J. C. (1985) *J. Biol. Chem. 260*, 202–207.

Cross, R. A., Bardsley, R. G., Ledward, D. A., Small, J. V., & Sobieszek, A. (1984) *Eur. J. Biochem. 145*, 305–310.

dos Remedios, C. D., & Cooke, R. (1984) *Biochim. Biophys. Acta 788*, 193–205.

Elzinga, M., & Collins, J. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4281–4284.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.

Gergely, J., & Seidel, J. C. (1983) in *Handbook of Physiology* (Peachey, L. D., & Adrian, R. H., Eds.) pp 257–274, American Physiological Society, Bethesda, MD.

Ikebe, M., & Hartshorne, D. J. (1984) *J. Biol. Chem. 254*, 11 638–11 642.

Ikebe, M., & Hartshorne, D. J. (1985) *Biochemistry 24*, 2380–2386.

Kielley, W. W., & Bradley, L. (1956) *J. Biol. Chem. 218*, 653–659.

Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.

Lu, R. C., & Lehrer, S. S. (1984) *Biochemistry 23*, 5975–5981.

Marsh, D. J., & Lowey, S. (1980) *Biochemistry 19*, 774–784.

Morales, M. F., Borejdo, J., Botts, J., Cooke, R., Mendelson, R. A., & Takashi, R. (1982) *Annu. Rev. Phys. Chem. 33*, 319–351.

Okamoto, Y., & Sekine, T. (1980) *J. Biochem. (Tokyo) 87*, 167–178.

Okamoto, Y., Okamoto, M., & Sekine, T. (1980) *J. Biochem. (Tokyo) 88*, 361–371.

Onishi, H. (1985) *J. Biochem. (Tokyo) 98*, 81–86.

Onishi, H., & Wakabayashi, T. (1982) *J. Biochem. (Tokyo) 92*, 871–879.

Onishi, H., & Watanabe, S. (1985) *Adv. Biophys. 19*, 21–42.

Reisler, E., Burke, M., Himmelfarb, S., & Harrington, W. F. (1974) *Biochemistry 13*, 3837–3840.

Seidel, J. C. (1978) *Biochem. Biophys. Res. Commun. 85*, 107–113.

Seidel, J. C. (1979) *Biochem. Biophys. Res. Commun. 89*, 958–964.

Seidel, J. C. (1980) *J. Biol. Chem. 255*, 4355–4361.

Sekine, T., & Yamaguchi, M. (1963) *J. Biochem. (Tokyo) 54*, 196–198.

Sobieszek, A., & Small, J. V. (1976) *J. Mol. Biol. 102*, 75–92.

Sutoh, K., Sutoh, R., Karr, T., & Harrington, W. F. (1978) *J. Mol. Biol. 126*, 1–22.

Suzuki, H., Onishi, H., Takahashi, K., & Watanabe, S. (1978) *J. Biochem. (Tokyo) 84*, 1529–1542.

Suzuki, H., Kamota, T., Onishi, H., & Watanabe, S. (1982) *J. Biochem. (Tokyo) 91*, 1699–1706.

Suzuki, H., Wong, A., Lu, R. C., & Seidel, J. C. (1984) *Biophys. J. 42*, 43a.

Suzuki, H., Stafford, W. F., Slayter, H. S., & Seidel, J. C. (1985) *J. Biol. Chem. 260*, 14 810–14 817.

Takashi, R., Muhlrad, A., & Botts, J. (1982) *Biochemistry 21*, 5661–5668.

Tao, T., & Lamkin, M. (1981) *Biochemistry 20*, 5051–5055.

Trybus, K. M., Huiatt, T. W., & Lowey, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6151–6155.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Weeds, A. G., & Pope, B. (1977) *J. Mol. Biol. 111*, 129–151.

Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970.

Wells, J. A., Knoeber, C., Sheldon, M. C., Werber, M., & Yount, R. G. (1980) *J. Biol. Chem. 255*, 11 135–11 140.

Yamashita, T., Soma, Y., Kobayashi, S., Sekine, T., Titani, K. Narita, K. (1964) *J. Biochem. (Tokyo) 55*, 576–578.