Nabel, E. G., Berk, B. C., Brock, T. A., & Smith, T. W. (1988) *Circ. Res. 62*, 486-493.
Nelson, M. T., & Blaustein, M. P. (1981) *Nature 289*, 314-316.
Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1982) *J. Biol. Chem. 256*, 11804-11808.
Nishimura, J., Kanaide, H., & Nakamura, M. (1986) *J. Pharmacol. Exp. Ther. 236*, 789-793.
Ozaki, H., & Urakawa, N. (1979) *Naunyn-Schmiedeberg's Arch. Pharmacol. 309*, 171-178.
Ozaki, H., & Urakawa, N. (1981) *Pfluegers Arch. 390*, 107-112.
Reeves, J. P. (1985) *Curr. Top. Membr. Transp. 25*, 77-127.
Reeves, J. P., & Sutko, J. L. (1980) *Science 208*, 1461-1464.
Reeves, J. P., & Hale, C. C. (1984) *J. Biol. Chem. 259*, 7733-7734.
Reeves, J. P., & Poronnik, P. (1987) *Am. J. Physiol. 252*, C17-C23.
Reuter, H., Blaustein, M. P., & Haeusler, G. (1973) *Philos. Trans. R. Soc. London B 265*, 87-94.
Sakai, V., Grover, A. K., Fox, J. E. T., & Daniel, E. E. (1983) *Can. J. Physiol. Pharmacol. 61*, 699-704.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502-514.
Slaughter, R. S., Sutko, J. L., & Reeves, J. P. (1983) *J. Biol. Chem. 258*, 3183-3190.
Slaughter, R. S., Welton, A. F., & Morgan, D. W. (1987a) *Biochim. Biophys. Acta 904*, 92-104.
Slaughter, R. S., Garcia, M. L., King, V. F., & Kaczorowski, G. J. (1987b) *Biophys. J. 51*, 386a.
Slaughter, R. S., Garcia, M. L., Cragoe, E. J., Jr., Reeves, J. P., & Kaczorowski, G. J. (1988) *Biochemistry 27*, 2403-2409.
Smith, J. B., & Smith, L. (1987) *J. Biol. Chem. 262*, 17455-17460.
Smith, J. B., Cragoe, E. J., & Smith, L. (1987) *J. Biol. Chem. 262*, 11988-11994.
Wibo, M., & Godfraind, T. (1984) *Arch. Int. Pharmacodyn. Ther. 270*, 333-334.
Wuytack, F., De Schutter, G., & Casteels, R. (1981) *Biochem. J. 198*, 215-271.

Isolation and Characterization of the N-Terminal 23-Kilodalton Fragment of Myosin Subfragment 1†

Andras Muhlrad*
Cardiovascular Research Institute, University of California, San Francisco, California 94143, and Department of Oral Biology, Hebrew University-Hadassah School of Dental Medicine, Jerusalem 91010, Israel

Received August 4, 1988; Revised Manuscript Received December 2, 1988

ABSTRACT: The 23-kDa N-terminal tryptic fragment was isolated from the heavy chain of rabbit skeletal myosin subfragment 1 (S-1). The heavy-chain fragments were dissociated by guanidine hydrochloride following limited trypsinolysis, and the 23-kDa fragment was isolated by gel filtration and ion-exchange chromatography. Finally, the fragment was renatured by removing the denaturants. The CD spectrum of the renatured fragment shows the presence of ordered structure. The tryptophan fluorescence emission spectrum of the fragment is considerably shifted to the red upon adding guanidine hydrochloride which indicates that the tryptophans are located in relatively hydrophobic environments. The two 23-kDa tryptophans, unlike the rest of the S-1 tryptophans, are fully accessible to acrylamide as indicated by fluorescence quenching. The isolated 23-kDa fragment cosediments with F-actin in the ultracentrifuge and significantly increases the light scattering of actin in solution which indicates actin binding. The binding is rather tight (K_d = 0.1 μM) and ionic strength dependent (decreasing with increasing ionic strength). ATP, pyrophosphate, and ADP dissociate the 23-kDa-actin complex with decreasing effectiveness. The isolated 23-kDa fragment does not have ATPase activity; however, it inhibits the actin-activated ATPase activity of S-1 by competing presumably with S-1 for binding sites on actin. F-Actin binds to the 23-kDa fragment immobilized on the nitrocellulose membrane. The fragment was further cleaved, and one of the resulting peptides, containing the 130-204 stretch of residues, was found to bind actin on the nitrocellulose membrane, indicating that this region of the 23-kDa fragment participates in forming an actin binding site.

Myosin subfragment 1 (S-1),[1] the head segment of myosin, contains separate actin and nucleotide binding sites of the molecule. These sites are involved in ATPase activity and actin-myosin interactions which are believed to have central significance in the molecular mechanism of muscle contraction. In order to understand the mechanism of these contraction events and the communication between the actin and nucleotide binding sites, it is necessary to define the S-1 structure.

The structure of S-1 heavy chain has been thoroughly studied by limited proteolytic digestion. Balint et al. (1978) found that the chain consists of three trypsin-resistant fragments (23, 50, and 20 kDa) aligned from the N-terminus in

† This research was supported by Grant 00241/85 from the US-Israel Binational Research Foundation, by Grant HL-16683, and by a grant from the Muscular Dystrophy Association.

* Address correspondence to this author at the Department of Oral Biology, Hebrew University-Hadassah School of Dental Medicine, Jerusalem 91010, Israel.

[1] Abbreviations: S-1, chymotryptic subfragment 1; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; ϵ-ATP, 1,N^6-ethenoadenosine 5′-triphosphate; PP_i, pyrophosphate; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kDa, kilodalton(s); NCS, *N*-chlorosuccinimide; DTE, dithioerythritol; DTT, dithiothreitol; BSA, bovine serum albumin; MeOH, methanol; TMR, tetramethylrhodamine iodoacetamide; Trp, tryptophan.

0006-2960/89/0428-4002$01.50/0

this order. The formation of stable proteolytic fragments provided for a convenient framework for the localization of various functionalities of S-1. Both the 23- and 50-kDa fragments appear to be involved in forming the ATP binding site (Szilagyi et al., 1979; Mahmood & Yount, 1984; Okamoto & Yount, 1985; Chaussepied et al., 1986a; Grammer et al., 1988). Finally, it was shown by cross-linking studies that the N-terminal region of 20 kDa and a stretch of residues on 50 kDa, located within 5 kDa from the C-terminus, constitute a part of the myosin–actin interface (Mornet et al., 1981; Sutoh, 1983).

The observation that tryptic digestion of S-1 results in three large protease-resistant fragments led to the suggestion that the tryptic fragments are domains formed by the S-1 heavy chain. These domains would appear to be connected to each other by short trypsin-susceptible "connectors" (Mornet et al., 1981). This domain hypothesis was further supported by the findings of Applegate and Reisler (1983) and Mornet et al. (1984), who showed that a number of proteases cleave S-1 into essentially the same fragments as does trypsin. However, the foregoing observations, though necessary, were not sufficient to prove the existence of domains in the S-1 structure, since it would be possible that the peptide chains of the various fragments interweave to form a common structure, leaving two unstructured regions exposed to proteolysis. Since domains are considered independently folded entities, we thought that by isolating the various S-1 fragments and studying their structure and function separately we could examine the validity of the "domain hypothesis" more rigorously. The isolation of tryptic fragments is rather problematic, because even after the peptide bonds are severed, the fragments remain associated by secondary forces, and their dissociation can be achieved only be denaturants (high concentration of urea, guanidine hydrochloride, or SDS). Therefore, the isolation, which is carried out in the presence of denaturants, has to be followed by renaturation, in the absence of denaturants. We recently isolated, renatured, and characterized the structure and function of the 20- and 50-kDa fragments of the S-1 heavy chain and found that both fragments contain actin binding sites (Muhlrad & Morales, 1984; Muhlrad et al., 1986). Chaussepied et al. (1986b,c) isolated various C-terminal fragments (20, 22, and 30 kDa) of S-1 complexed with LC_3 alkali light chain and observed that this complex interacts with actin. Morita and co-workers described the interaction between the isolated 20-kDa fragment and the LC_1 light chain (Ueno et al., 1985) and showed that not only the N-terminal region of the 20-kDa domain but also the stretch between the SH_1 and SH_2 thiols interacts with actin (Katoh & Morita, 1984; Katoh et al., 1985; Suzuki et al., 1987).

Here we present for the first time the isolation, preparation, and functional characterization of the renatured 23-kDa N-terminal fragment of S-1. This fragment has special significance, because it contains the nucleotide binding site of myosin (Szilagyi et al., 1979; Okamoto & Yount, 1985; Grammer et al., 1988) and because, according to the results below, it may participate in actin binding as well. Therefore, it is quite possible that the 23-kDa fragment has a key role in the process of energy transduction, which is the molecular basis of muscle contraction. A preliminary report of this work has been published (Muhlrad, 1988).

Materials and Methods

Reagents. Ultrapure urea, sucrose, and guanidine hydrochloride were purchased from Schwarz/Mann. Sephadex G-100 and QAE-Sephadex G-50 were from Pharmacia. AG1-X2 ion-exchange resin, SDS, and electrophoresis reagents were from Bio-Rad. TMR was bought from Molecular Probes. ATP, ADP, AMP, PMSF, and NCS were best-grade products of Sigma. TPCK–trypsin and α-chymotrypsin were from Worthington. Leupeptin was from Calbiochem. Alkaline phosphatase conjugates of anti-mouse and anti-rabbit IgG and alkaline phosphatase substrates were from KPL. All other chemicals were of reagent grade. The mAb.25 (anti-50 kDa) and the N3.36 (anti-23 kDa) monoclonal antibodies are in use in our laboratory, and the anti-20-kDa polyclonal antibody was raised against isolated 20-kDa fragment.[2]

Proteins. Myosin and actin were prepared from back and leg muscles of rabbit according to Tonomura et al. (1966) and Spudich and Watt (1971), respectively. S-1 was prepared by digesting myosin filaments with α-chymotrypsin (Weeds & Taylor, 1975). Myosin, S-1, and 23-kDa fragment concentrations were estimated by assuming $A^{1\%}_{280nm}$ = 5.7, 7.5, and 14.3, respectively, and $A^{1\%}_{290nm}$ = 6.3 for G-actin. The $A^{1\%}_{280nm}$ for the isolated 23-kDa fragment was obtained by measurement of its amino acid content in the HCl hydrolysate or from its tryptophan concentration (two tryptophans per fragment) by measuring the fluorescence intensity in the presence of 6 M guanidine hydrochloride. In all absorbance measurements, light-scattering corrections were applied.

CD Spectrum. This was recorded in a JASCO J-500A spectropolarimeter, under nitrogen in a thermostated cell holder, at 20 °C. Various path-length-fused silica cells were used.

Fluorescence Measurements. These were made with an SLM 8000 spectrofluorometer interfaced with an IBM-XT computer. All emission spectra were corrected for bakcground, buffer, and scattered light.

Acrylamide quenching of tryptophan fluorescence was performed by adding 5-μL aliquots of 3 M acrylamide to the sample at 15 °C and by averaging the fluorescence signal for 30 s following each addition. Maximal acrylamide concentration used was 100 mM; at this concentration, no absorbance correction was necessary. Excitation and emission wavelengths were 295 and 330 nm, respectively. The sample, which was light sensitive, was illuminated only during the time required for the measurement.

ATPase Assay. ATPase activities (micromoles of P_i per milligram of S-1 per minute) were calculated from the production of inorganic phosphate (P_i) using a Fiske–Subbarow method. The reaction was carried out at 25 °C. K^+-(EDTA)-activated ATPase was measured in samples containing 2 μM 23-kDa fragment, 2 mM ATP, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0, and 600 mM KCl. Actin-activated S-1 ATPase was measured in samples containing 0.1 μM S-1, 0.5 μM F-actin, various concentrations of 23-kDa fragment, 1 mM ATP, 2 mM $MgCl_2$, and 20 mM imidazole hydrochloride buffer, pH 7.0. The incubation time was chosen so that not more than 15% of the ATP added was hydrolyzed.

Light Scattering. The intensity of the light scattered at 90° to incidence was measured in a silica cell in an SLM 8000 spectrofluorometer in a constant-temperature cell holder at 20 °C. Both excitation and emission monochromators were set at 400 nm.

Sedimentation. The 23-kDa fragment was mixed F-actin and sedimented in a preparative ultracentrifuge. The super-

[2] mAb.25 and N3.36 monoclonal antibodies were developed against human skeletal myosin; their epitopes were located within the 50- and 23-kDa fragments of the S-1 heavy chain, respectively (Dan-Goor, Silberstein, Kessel, and Muhlrad, submitted for publication). Anti-20-kDa polyclonal antibody was raised in rabbit against 20-kDa fragment isolated from chicken skeletal S-1 (Muhlrad and Groschel-Stewart, unpublished results).

natant was separated from the pellet and analyzed by SDS–PAGE. The Coomassie blue stained slab gels were quantitatively evaluated by densitometry. Appropriate controls (23-kDa fragment without F-actin and F-actin without 23-kDa fragment) were also run.

SDS–PAGE and Immunoblotting. Electrophoretic analysis of the samples was performed on 5–18% polyacrylamide gradient slab gels (Mornet et al., 1981). Molecular weights of the protein bands were estimated by comparing their electrophoretic mobilities with those of authentic markers. For immunoblots, protein bands were electrophoretically transfered from SDS–PAGE to nitrocellulose membrane (Burnette, 1981). The nitrocellulose membrane was cut into four parts; one part was stained by amido black, while three others were immunostained with mAb.25 (anti-50 kDa) monoclonal, N3.36 (anti-23 kDa) monoclonal, and anti-20-kDa polyclonal antibodies. The second antibodies were alkaline phosphatase conjugates of anti-mouse IgG and anti-rabbit IgG, used for the monoclonal and polyclonal antibodies, respectively. The reacting protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium alkaline phosphatase substrates.

Labeling of Actin. G-Actin (5.5 mg/mL, 130 μM) in 1 mM $KHCO_3$, 1 mM NaN_3, 0.1 mM $CaCl_2$, and 0.2 mM ATP, pH 7.6, was reacted with 140 μM TMR in the dark at 0 °C overnight. Unreacted reagent was quenched by addition of 2 mM DTE and removed by filtering the sample on a preprepared Sephadex G-25 column. Labeled actin was polymerized by 0.1 M KCl and dialyzed (one change) against 100 volumes of 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTE, 1 mM NaN_3, and 5 mM imidazole, pH 7.0, in the dark at 4 °C for 2 days. The biological properties (polymerizability, activation of S-1 ATPase) of the labeled actin were essentially identical with those of the native actin.

Tryptic Digestion of S-1. S-1 (7 mg/mL) was digested by TPCK–trypsin with 1% (w/w) of its concentration in 20 mM Tris-HCl, pH 7.8, at 25 °C for 20 min. Digestion was terminated by adding 1 mM PMSF and incubating for 20 min at 25 °C. Finally, 0.5 volume of protein sample buffer, containing 5% SDS, 2% β-mercaptoethanol, 20 mM Tris-HCl, pH 8.4, 40% glycerol, and 0.01% bromophenol blue, was added, and the mixture was allowed to stand at room temperature for at least 30 min before being applied to the SDS–polyacrylamide slab gel.

Chemical Cleavage of the 23-kDa Fragment by NCS. This was carried out essentially according to Rodney and Doolittle (1985). The method is based on the findings of Shechter et al. (1976), who showed that NCS cleaves peptide bonds selectively at tryptophan residues. Following tryptic digestion, S-1 was electrophoresed on SDS–PAGE. A Coomassie blue stained band of 23-kDa fragment was cut from the gel. The gel slices were then incubated in 20, 50, and 100% methanol successively and finally freeze-dried. The dried gel slices were soaked in 2 mL of 88% formic acid containing 0.5 mg/mL NCS at room temperature for 30 min. The reaction was quenched by adding 2 mL of 3 mM methionine. The gel slices were then washed with methanol, freeze-dried, the reapplied to SDS–PAGE as described above.

Nitrocellulose Overlay by Labeled F-Actin. S-1, its tryptic digest, and isolated 23-kDa fragment before and after NCS cleavage were electrophoresed on SDS–PAGE and transferred to nitrocellulose as described above. Following the transfer, the nitrocellulose was cut into two pieces. One piece was stained with amido black (0.1% amido black in 45% MeOH/10% acetic acid) for 10 min and then destained with 48% MeOH and 2% acetic acid for 10 min. The other piece was incubated for 1 h with shaking at room temperature, with 100 mL of blocking buffer containing 1% BSA in 154 mM NaCl and 10 mM Tris-HCl (Tris–saline), pH 7.4. This was followed by 4 × 10 min washings with 100 mL of Tris–saline and then incubating for 4 h, while being shaken at room temperature, with 35 mL of 1 μM TMR-labeled F-actin in 25 mM KCl, 1 mM $MgCl_2$, and 5 mM sodium phosphate buffer, pH 7.0. Finally, excess actin was removed by 4 × 10 min washings with 100 mL of Tris–saline. The actin-overlayed nitrocellulose membrane was air-dried and could be stored indefinitely. The binding of fluorescent F-actin was visualized by a UV transilluminator using a 365-nm filter.

Results

Isolation of the 23-kDa Fragment. In a typical experiment, 30 mL of 8 mg/mL S-1 in 30 mM Tris HCl, pH 7.8, was digested with TPCK–trypsin in a 100:1 (w/w) S-1 to trypsin ratio, at 25 °C, for 30 min. Digestion was terminated by adding soybean trypsin inhibitor in a 3:1 (w/w) ratio to trypsin. The digest was applied to a Sephadex G-100 column (2.6 × 90 cm) equilibrated with 30 mM Tris-HCl, pH 7.8, at 4 °C. The protein peak was pooled and treated with 6 M guanidine hydrochloride in the presence of 2 mM EDTA and 2 mM DTT at 25 °C for 30 min in order to dissociate the tryptic fragments of the S-1 heavy chain. Finally, the digest was cooled, and 2.5 volumes of −18 °C ethanol were added. It was mixed thoroughly with ethanol and was kept overnight at −18 °C. The 23-kDa fragment and partially the 50-kDa fragment were precipitated by the ethanol, and the precipitate was collected by sedimentation (25000*g*, 30 min, 4 °C). The pellet was washed 3 times by 4 mM β-mercaptoethanol/10 mM Tris–acetate, pH 7.8, and dissolved in 1.4 mL of 2.5% SDS, 4 mM β-mercaptoethanol, and 10 mM Tris–acetate, pH 7.8. The dissolved material was cleared by centrifugation (25000*g*, 15 min, 20 °C) and applied to a Sephadex G-100 column (1.6 × 90 cm, room temperature) equilibrated with 0.1% SDS, 4 mM β-mercaptoethanol, and 10 mM Tris-acetate, pH 7.8; 2.5-mL fractions were collected at an 8 mL/h flow rate. Two protein peaks obtained were analyzed by SDS–PAGE. The first peak contained mainly the 50-kDa fragment, and the main component in the second peak was the 23-kDa fragment plus some 20-kDa fragment and light-chain contaminations. The fractions containing the 23-kDa fragment were pooled and dialyzed against 200 mL of 6 M urea, 4 mM β-mercaptoethanol, 1 mM EDTA, and 50 mM Tris–acetate, pH 7.8, overnight at 4 °C. After dialysis, the remaining SDS was removed by an AG1-X2 anion-exchange column (0.9 × 4 cm, 25 °C) equilibrated with 6 M urea, 4 mM β-mercaptoethanol, and 50 mM Tris–acetate, pH 7.8. The protein-containing fractions were pooled again and applied to a QAE-Sephadex G-50 column (0.9 × 12 cm, 25 °C) equilibrated with 6 M urea, 4 mM β-mercaptoethanol, and 50 mM $NaHCO_3$, pH 8.2, in order to purify the 23-kDa fragment from contaminating peptides. The 23-kDa fragment was eluted from the column by 20 mM NaCl in the equilibrium buffer (Figure 1). One protein peak was obtained which according to SDS–PAGE (Figure 2) contained pure 23-kDa fragment (its apparent molecular weight on the basis of electrophoretic mobility was 27K). In order to renature the purified 23-kDa fragment, urea was removed by stepwise dialysis. The 15 mL of 23-kDa preparation was dialyzed first against 250 mL of 3 M urea, 0.2 M sucrose, 4 mM β-mercaptoethanol, and 50 mM Tris–acetate, pH 7.8. The second dialysis was against the same solution, but 1 M urea and 4 μg/mL leupeptin (protease inhibitor) were also added. The third dialyzing

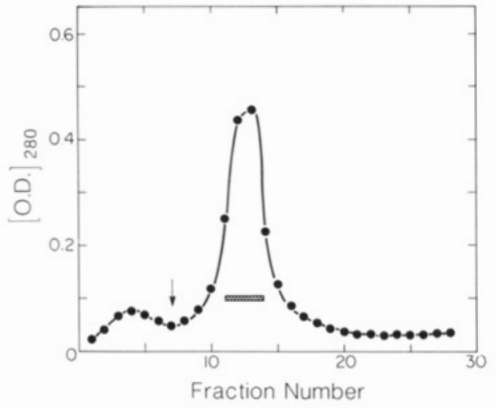

FIGURE 1: Purification of the 23-kDa fragment by ion-exchange chromatography. Crude 23 kDa in 6 M urea, 4 mM β-mercaptoethanol, and 50 mM Tris–acetate, pH 7.8, was applied to a QAE-Sephadex G-50 column (0.9 × 12 cm) equilibrated with 8 M urea, 4 mM β-mercaptoethanol, and 50 mM $NaHCO_3$, pH 8.2. Elution started at the arrow with 20 mM NaCl in the elution buffer. 2.5-mL fractions were collected, and OD_{280} was recorded. Fractions were analyzed by SDS-PAGE, and those labeled with the bar were pooled.

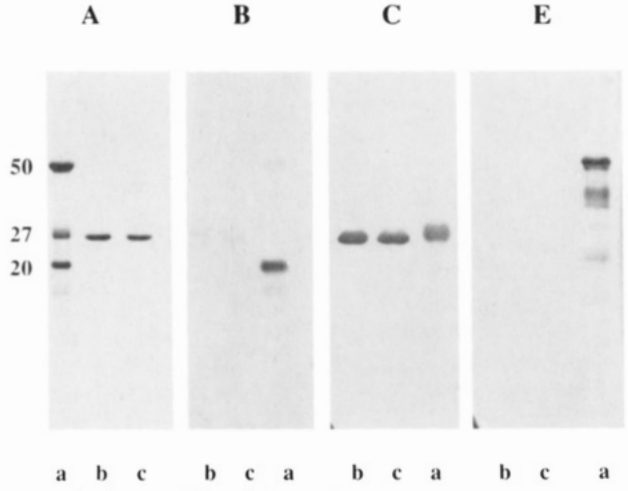

FIGURE 2: Immunoblot of the purified 23-kDa fragment. Trypsin-digested S-1 (a), pooled protein peak from the QAE-Sephadex G-50 column (b), and purified 23-kDa fragment after removal of urea (c) were analyzed by SDS–PAGE (A). Following SDS–PAGE, the bands were transferred to a nitrocellulose membrane and immunostained as described under Materials and Methods by anti-20-kDa polyclonal (B), anti-23-kDa monoclonal (C), and anti-50-kDa monoclonal (E) antibodies. Vertical numbers are the apparent molecular mass, based on electrophoretic mobility, in kilodaltons. 23 kDa has an anomalous electrophoretic mobility; it runs as 27 kDa. Therefore, the 27-kDa band in all SDS–PAGE gels corresponds to the 23-kDa fragment.

solution (500 mL) was the same as the second, but did not contain urea. All dialyzing steps were carried out at 4 °C for about 12 h; 0.2 M sucrose was added to the dialyzing solution in order to decrease the tendency of the isolated fragment to aggregate. [We found earlier that the isolated 20-kDa fragment aggregates easily (Muhlrad et al., 1986) and that sucrose considerably reduces its tendency to aggreate.] Following dialysis, the aggregated material was removed by centrifugation (150000g at 4 °C for 1 h). It is worth mentioning that the tendency of the 23-kDa fragment to aggregate is significantly less than that of the 20-kDa fragment; 15 mL of 140 μg/mL purified 23-kDa fragment was obtained (altogether 2.1 mg from 240 mg of S-1). The isolated and renatured 23-kDa preparations were kept on ice and used only for 3–4 days in different experiments. The purity of the 23-kDa preparation was assessed by electrophoresis and immunoblotting, using three antibodies reacting specifically with the

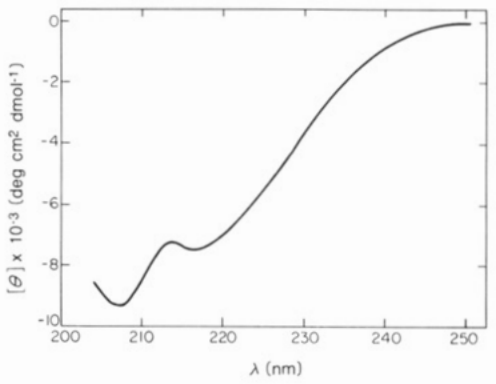

FIGURE 3: Circular dichroism (CD) spectrum of the isolated 23-kDa fragment. 1.8 μM 23-kDa fragment, in 50 mM sucrose, 0.5 mM DTT, and 5 mM sodium phosphate, pH 7.0.

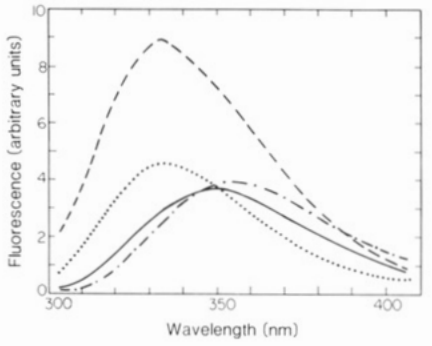

FIGURE 4: Fluorescence spectra of 23 kDa and S-1 in the absence and presence of guanidine hydrochloride. Excitation wavelength was 295 nm. Isolated 23-kDa fragment, 4 μM, (···) in the absence and (—) in the presence of 6 M guanidine hydrochloride. S-1, 1.6 μM, (- - -) in the absence and (—) in the presence of 6 M guanidine hydrochlorides. (The spectra of 23 kDa and S-1 in the presence of 6 M guanidine hydrochloride were identical.) *N*-Acetyltryptophanamide, 8 μM (–·–). In all cases, Trp concentration was 8 μM (S-1 contains five and 23 kDa two Trp's). Samples were in 0.2 M sucrose, 1 mM DTT, and 5 mM sodium phosphate, pH 7.0.

23-, 50-, and 20-kDa domains of the S-1 heavy chain, respectively (Figure 2). The isolated 23-kDa fragment reacted exclusively with the anti-23-kDa antibody, giving only a single Coomassie blue stained band following SDS–PAGE on the gradient gel. The amino acid composition of the isolated 23-kDa fragment (data not shown) was in good agreement with the expected composition calculated from the known amino acid composition of the fragment (Tong & Elzinga, 1983).

Structural Characterization of the Isolated 23-kDa Fragment. The CD spectrum of the 23-kDa fragment was measured between 204 and 250 nm (Figure 3). Analysis of the spectrum (Chang et al., 1976) revealed that the renatured fragment contains 20% α-helix, 35% β-sheet, and 12% β-turn. These values are somewhat lower than those estimated (30% α-helix, 42% β-sheet, and 13% β-turn) from the known amino acid sequence of 23-kDa fragment (Tong & Elzinga, 1983) according to Chou and Fasman (1974). Addition of 6 M guanidine hydrochloride or 8 M urea made the spectrum more flat (not shown), pointing to a loss of ordered structure.

The 23-kDa fragment contains two of the five S-1 Trp's (the rest reside on the 50-kDa domain). The Trp fluorescence spectrum of the 23-kDa fragment was measured and compared to that of S-1 (Figure 4). The maximum of the emission spectrum of the renatured 23-kDa fragment was similar to that of native S-1 (334 and 332 nm, respectively) except for a slight red shift. However, the Trp fluorescence intensity of the 23-kDa fragment was smaller than that of S-1. Addition of

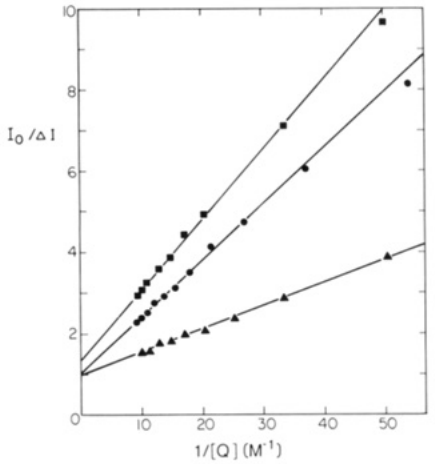

FIGURE 5: Acrylamide quenching of Trp fluorescence of the isolated 23-kDa fragment. Modified Stern–Volmer plot (Lehrer, 1971). All samples in 0.2 M sucrose, 1 mM DTT, and 5 mM sodium phosphate, pH 7.0. 23-kDa fragment, 4 μM (●); S-1, 2 μM (■); *N*-acetyltryptophanamide, 8 μM (▲). Abscissa, reciprocal concentration of acrylamide, 1/[Q] M^{-1}; ordinate, fluorescence intensity of the unquenched sample (I_0)/decrease in intensity upon addition of acrylamide (ΔI). The quenching constant, K_{SV}, and the fraction of tryptophan residues accessible to the quencher, f_a, were calculated from the plot according to the equation: $I_0/\Delta I = 1/f_a + 1/f_a K_{SV}[Q]$.

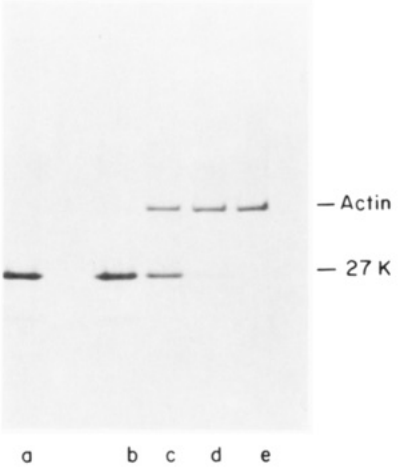

FIGURE 6: Binding of 23-kDa fragment to actin analyzed by cosedimentation. To 3 μM 23-kDa fragment was added 3 μM F-actin in 0.2 M sucrose, 25 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7.0, with or without 3 mM ATP. Samples were centrifuged at 150000*g* for 45 min at 4 °C. The supernatants were analyzed by SDS–PAGE. (a) 23 kDa without actin before centrifugation; (b) 23 kDa supernatant, no actin; (c) actin–23-kDa supernatant with ATP; (d) actin–23-kDa supernatant, no ATP; (e) actin supernatant, no 23 kDa.

6 M guanidine hydrochloride induced a strong red shift and loss of fluorescence intensity in the spectra of both 23 kDa and S-1, but the loss of intensity in S-1 was greater than in 23 kDa.

The fluorescence of the 23-kDa Trp's is quenchable by acrylamide (Figure 5). The modified Stern–Volmer plot for quenching (Lehrer, 1971) is linear and can be characterized by a single Stern–Volmer constant, K_{SV} = 7.8 M^{-1}. Under identical conditions, K_{SV} values of the accessible Trp's of S-1 and of the model compound *N*-acetyltryptophanamide are 8.2 and 16.8 M^{-1}, respectively. The intercepts on the ordinate, which gives the reciprocal of the fraction of the acrylamide quenchable fluorescence ($1/f_a$), are 1.02 and 1.35 for 23 kDa and S-1, respectively. This indicates that, unlike some other S-1 Trp's, both Trp's of the 23-kDa fragment are accessible to acrylamide.

Interaction of the Isolated 23-kDa Fragment with Actin and Nucleotide. The interaction of the isolated 23-kDa fragment with actin was studied by cosidementing the fragment with F-actin in the ultracentrifuge (Figure 6). When the 23-kDa fragment was cosedimented with actin in the absence of MgATP (lane d), essentially no 23 kDa remained in the supernatant, while without actin (lane b) no 23 kDa was sedimented. This indicates that the two proteins interacted with each other. When 23 kDa was cosedimented with actin in the presence of MgATP (lane c), about 50% of 23 kDa did not sediment, which shows that the 23-kDa–F-actin interaction is ATP dependent and ATP is capable of dissociating the complex of the two proteins.

The interaction between the 23-kDa fragment and actin was also characterized by light-scattering measurements. A significant increase in light scattering was observed upon mixing the 23 kDa with F-actin, and the increment was much higher than the sum of the light scattering of the two protein solutions. The 23-kDa fragment was titrated with F-actin, and the observed light-scattering increment was plotted against the actin concentration (Figure 7). The titration curve reached a plateau and was used to estimate the affinity and the stochiometry of the reaction between the 23-kDa fragment and actin. The K_{assoc} and the stochiometry (n) were calculated from a Scatchard plot as 1.25 × 10^7 M^{-1} and 1.08, respectively,

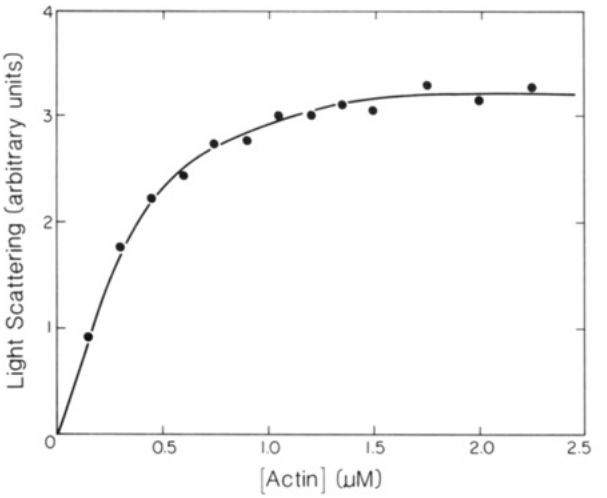

FIGURE 7: Binding of 23-kDa fragment to actin: a light-scattering measurement. To 0.5 μM 23-kDa fragment in 0.2 M sucrose, 1 mM $MgCl_2$, 25 mM KCl, 1 mM DTT, and 5 mM sodium phosphate, pH 7.0, 20 °C, was added F-actin in 0.15 μM increments, and the change in light scattering at 400 nm was recorded. Ordinate: light scattering observed upon addition of F-actin minus light scattering of F-actin alone, in arbitrary units.

which indicates a tight binding with a 1:1 stoichiometry between the two proteins.

Since the sedimentation experiments showed that the binding of the 23-kDa fragment to actin is ATP dependent, we used light scattering to characterize quantitatively the effect of ATP and its analogues on the 23-kDa–actin interaction. To a mixture of 23-kDa fragment of F-actin, ATP or its analogues were added, in the presence or absence of Mg; then the change in the light-scattering intensity was recorded (Figure 8). It was found that ATP more effectively dissociates the 23-kDa–actin complex in the absence of Mg than in its presence. Of all the dissociating agents studied, ATP was the most effective followed by PP_i, ADP, and AMP. Relatively high concentrations of AMP, 8–10 mM, had to be added to get some appreciable dissociation. The apparent affinity of ATP to the 23-kDa fragment was estimated from the concentration of ATP needed to cause 50% dissociation of the 23-kDa–actin

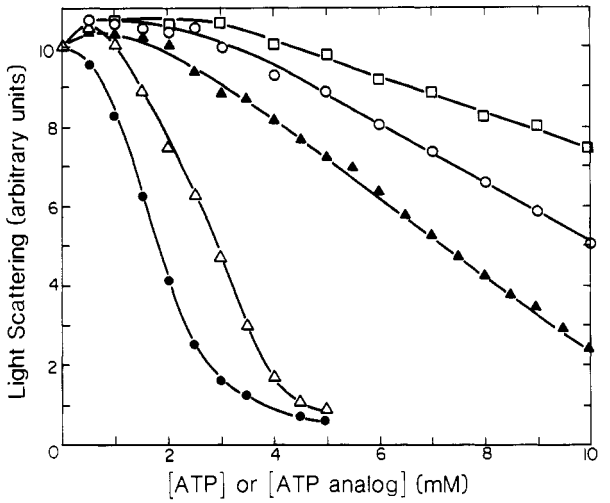

FIGURE 8: Dissociation of the 23-kDa–actin complex by ATP or its analogues: light-scattering measurements. To a mixture of 1 μM 23-kDa fragment and 1 μM F-actin in 0.2 M sucrose, 1 mM DTT, 25 mM KCl, 0.5 mM EDTA, and 5 mM sodium phosphate, pH 7.0, 20 °C, were added ATP or ATP analogues in 0.5 mM increments, and the change in light scattering at 400 nm was recorded. ATP was also added in the presence of 1 mM $MgCl_2$ in the absence of EDTA. ATP with EDTA (●); ATP with $MgCl_2$ (△); PP_i (▲); ADP (○); AMP (□). Ordinate: light scattering observed minus light scattering of F-actin alone, in arbitrary units.

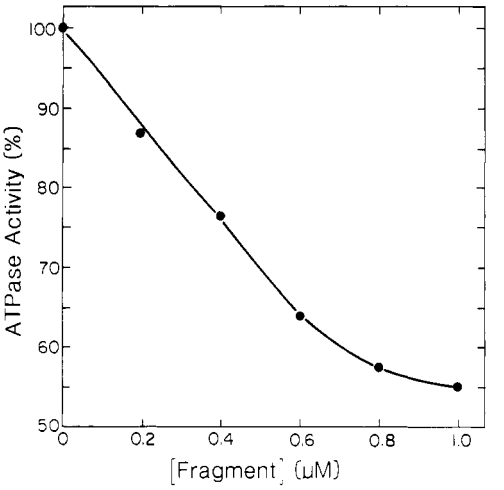

FIGURE 9: Effect of 23-kDa fragment on the actin-activated ATPase activity of S-1. 23 kDa was added to the reaction mixture containing 0.1 μM S-1 and 0.5 μM F-actin as indicated on the abscissa, and ATPase activity was measured as described under Materials and Methods. Ordinate: ATPase activity expressed as percent of activity in the absence of 23 kDa (0.6 mol of P_i (mol of S-1)$^{-1}$ s^{-1}).

complex as K_{assoc} = 0.55 × 10^3 and 0.34 × 10^3 M^{-1} in the absence and presence of Mg, respectively.

The interaction between ATP and the 23-kDa fragment was studied also more directly without involving actin. First it was checked whether the fragment has ATPase activity, since the adenine ring binding site of ATP is supposed to reside on this fragment in the native S-1 (Szilagyi et al., 1979; Okamoto & Yount, 1985). Not even traces of ATPase activity were found when it was measured either with K^+(EDTA) or with Mg^{2+} as activator. No binding of ϵ-ATP (a fluorescence analogue of ATP) was observed (data not shown) when 4 μM 23-kDa fragment in 0.2 M sucrose, 5 mM sodium phosphate, pH 7.0, 200 mM acrylamide, and 1 mM DTT was titrated with 6 μM ϵ-ATP (maximal concentration). [This method (Ando et al., 1982) is based on the finding that the fluorescence of tightly bound ϵ-ATP, or ϵ-ADP, cannot be quenched by acrylamide.] The lack of ϵ-ATP binding in the micromolar range indicates that the association constant must be less than 10^6 M^{-1} and this is in agreement with Figure 8, which showed that ATP is needed in millimolar concentration to dissociate the 23-kDa–actin complex. Finally, the effect of ATP on the intrinsic Trp fluorescence of 23 kDa was studied. A decrease of about 5% in fluorescence intensity was observed when ATP, ADP, or PP_i was added to the 23-kDa fragment, either in the presence or in the absence of 1 mM $MgCl_2$ (2.5 μM 23-kDa fragment in 0.2 mM sucrose, 1 mM DTT, and 5 mM sodium phosphate, pH 7.0; excitation 295 nm, emission 330 nm) (data not shown). The decrease in fluorescence intensity was reproducible but too small to obtain reliable titration curves with increasing amounts of nucleotides.

In the following experiment, the effect of ionic strength on the 23-kDa–actin interaction was studied. Increasing concentrations of KCl were added to a mixture of 1 μM 23-kDa fragment and 1 μM F-actin in 0.2 M sucrose, 25 mM KCl, 1 mM DTT, 0.5 mM EDTA, and 5 mM sodium phosphate, pH 7.0, and the change in light scattering was recorded (data not shown). The light scattering of the solution started to decrease at 50 mM, reduced to half at 120 mM, and the dissociation of the 23-kDa–actin complex was practically complete at 160 mM KCl concentration.

Finally, the competition between the 23-kDa fragment and S-1 for actin was studied by measuring the effect of the fragment on the actin-activated ATPase of S-1 (Muhlrad & Morales, 1984). To a reaction mixture containing S-1 and F-actin was added 23-kDa fragment in increasing concentrations, and the ATPase activity was measured (Figure 9). The fragment inhibited the actin-activated ATPase activity of S-1; 5 μM 23 kDa caused 45% inhibition. The inhibition indicates either that S-1 cannot bind to the complex of actin and 23-kDa fragment or that the complexation with 23 kDa prevents the proper activation of S-1 ATPase by actin. The reason why the inhibition is not total even at a 2:1 23-kDa to actin ratio is the significant decrease of affinity between the two proteins in the presence of MgATP (see Figures 6 and 8).

Localization of the Actin Binding Site of the 23-kDa Fragment. The actin binding of S-1 and its tryptic fragments was also studied by a new actin overlay method (Muhlrad & Kessel, 1987). S-1 or its tryptic fragments were electrophoresed on SDS–PAGE and then electrically transferred to a nitrocellulose membrane which was overlaid by TMR-labeled F-actin (Figure 10). Actin reacted with the S-1 heavy chain and slightly with the LC_1 light chain (lane d). After tryptic digestion of S-1, the 20- and 23-kDa (according to electrophoretic mobility, 27 kDa) fragments were reacted by actin while the 50-kDa fragment and the marker proteins did not give any reaction (lanes e and f).

In order to localize the actin binding site on the 23-kDa fragment, the isolated fragment was partially cleaved by NCS (for details, see Materials and Methods) which cuts peptide bonds specifically at the carboxyl groups of tryptophans (Schechter et al., 1976). The NCS-cleaved 23-kDa fragment was applied to SDS–PAGE and transferred to nitrocellulose, which was then overlaid by TMR-labeled F-actin (Figure 11). Four major NCS peptides with apparent molecular masses of 18, 15, 11, and 8 kDa were obtained, and it was possible to assign the peptides in the known amino acid sequence of 23 kDa (Tong & Elzinga, 1983) by relating their molecular masses to the positions of the two tryptophans (Trp-112 and Trp-130) in the primary structure of the 23-kDa fragment, which contains 204 residues. In this way, the 18-kDa peptide was assigned to the 1–130, the 15-kDa peptide to the 1–112,

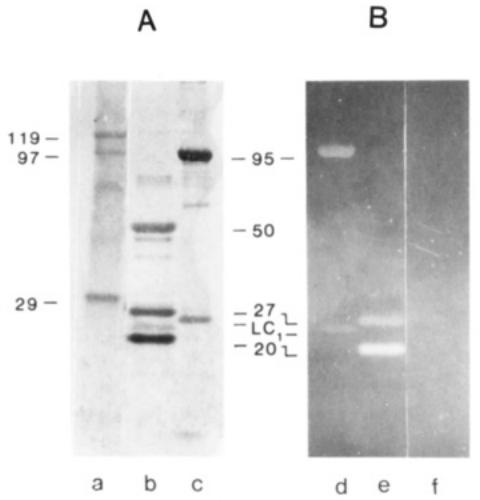

FIGURE 10: Overlay of S-1 and S-1 fragments by TMR-labeled F-actin on nitrocellulose. For procedures, see Materials and Methods. Amido black stain (A); TMR–actin fluorescence following overlay (B). (a and f) Marker proteins (β-galactosidase, phosphorylase, and carbonic anhydrase; molecular masses 119 kDa, 97 kDa, and 29 kDa, respectively); (b and e) trypsin-digested S-1; (c and d) S-1. Vertical numbers: apparent molecular mass, based on electrophoretic mobility, in kilodaltons.

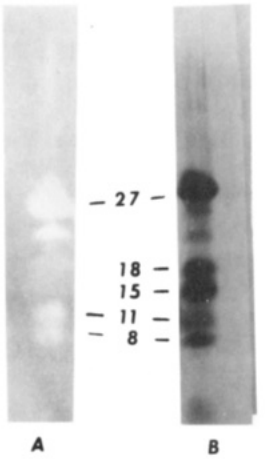

FIGURE 11: Overlay of NCS peptides of the 23-kDa fragment by TMR-labeled F-actin on nitrocellulose. For procedures, see Materials and Methods. Nitrocellulose membrane overlaid by fluorescent TMR–actin (A). Peptide bands stained by amido black (B). Vertical numbers: apparent molecular mass, based on electrophoretic mobility, in kilodaltons.

the 11-kDa peptide to the 112–204, and the 8-kDa peptide to the 130–204 amino acid residue stretch, respectively. Since actin reacted mainly with the 11- and 8-kDa peptides, which contain the C-terminal part of the 23-kDa fragment, one may conclude that an actin binding site resides on the C-terminal 130–204 stretch of the 23-kDa fragment.

Discussion

The N-terminal 23-kDa fragment of the S-1 heavy chain was isolated for the first time from the tryptic digest of S-1 following dissociation of the heavy-chain fragments in guanidine hydrochloride. A similar procedure was employed for the isolation of the other two heavy-chain fragments (Muhlrad & Morales, 1984; Muhlrad et al., 1986; Ueno et al., 1985). The solubility of the 23-kDa fragment differs from that of the other two, as it was precipitated by ethanol in the presence of guanidine hydrochloride, while the 50-kDa fragment remained partially, and the 20 kDa completely, soluble. This solubility difference was used to separate 23 kDa from the rest of the fragments in the first purification step. Additional steps, gel filtration and ion-exchange chromatography, were needed in order to get pure 23-kDa preparation, and it was necessary to remove the denaturants to refold the fragment. This was carried out in the presence of sucrose, which seems to stabilize the structure and decrease the aggregation of the preparation. It was difficult to avoid the degradation of the 23-kDa fragment during the preparation, since a protease (most probably trypsin) was tightly attached to the fragment. In order to prevent degradation, we removed trypsin after digestion of S-1 by gel filtration and added the protease inhibitor leupeptin to the purified fragment during and after removal of urea.

The CD spectrum which was recorded only between 204 and 250 nm, because of the presence of sucrose, indicated a considerable amount of ordered secondary structure in the purified 23-kDa fragment. This structure was greatly reduced by addition of urea or guanidine hydrochloride.

The 23-kDa fragment is known to contain two of the five S-1 Trp's (Tong & Elzinga, 1983). The Trp fluorescence emission spectrum of the isolated 23-kDa fragment resembles that of S-1, indicating that the 23-kDa Trp's are located in relatively hydrophobic environments. Such environments can be explained either by proximity of ordered secondary structures to the indole moiety of Trp's or by aggregation of the fragment. In this latter case, the appearance of some heterogeneity would be expected in the spectral properties of the fragment. Such heterogeneity would be manifested as a shoulder in the spectrum, an increased bandwidth of the emission peak, or a nonlinear Stern–Volmer plot for the acrylamide quenching. No evidence for heterogeneity was found in these spectral properties; this seems to exclude the possibility that the relatively hydrophobic environments of the Trp's are due to the aggregation of the fragment.

The fluorescence emission of the five S-1 Trp's was resolved into three lifetime classes (small-, intermediate-, and long-lifetime component) on the basis of their decay-associated spectra (Torgerson, 1984). Only the fluorescence emission of the long-lifetime component was quenchable by acrylamide. In the present work, it was found that the fluorescence of both 23-kDa Trp's are quenchable by acrylamide and their quenching constant, K_{SV}, is similar to that of the acrylamide-quenchable Trp's of S-1 (Torgerson, 1984). The Trp emission spectrum of the 23-kDa fragment was slightly red shifted relative to the spectrum of S-1, and upon addition of guanidine hydrochloride to 23 kDa, smaller loss of fluorescence intensity was observed than in the case of S-1. All these observations indicate that the 23-kDa Trp's are less shielded from water and solute molecules than the rest of the Trp's in S-1. However, the possibility that in intact S-1 the 23-kDa Trp's are less exposed than in the isolated fragment because they are buried by the other two domains could be also considered. Our recent results showing that the 23-kDa Trp's are reactive also in intact S-1 (Werber et al., 1987) seem to weaken this possibility.

The adenine ring binding site of ATP is located on the 23-kDa fragment (Szilagyi et al., 1979; Okamoto & Yount, 1985; Atkinson et al., 1986). Therefore, it seemed of interest to study whether the renatured fragment has ATPase activity or binds ATP. No traces of ATPase were found, and no high-affinity binding of ϵ-ATP [as was seen with S-1 (Ando et al., 1982)] was observed; however, low-affinity binding of ATP (K_d in the millimolar range) was indicated by the dissociating effect of ATP on the 23-kDa–actin complex. The lack of ATPase activity and tight nucleotide binding is not surprising since at least the 50-kDa fragment is also involved in the formation of the ATP binding site as indicated by

cross-linking the ribose moiety of a photoaffinity ATP analogue (Mahmood & Yount, 1984) and by the fact that the partial unfolding of the 50-kDa fragment by mild heat treatment (Setton & Muhlrad, 1984; Burke et al., 1987; Setton et al., 1988) or by methanol (Burke & Sivaramakrishnan, 1986) abolishes the ATPase activity of S-1. The foregoing results imply that at least two domains of S-1 are involved in the formation of the catalytic site.

This is the first time that binding of the 23-kDa fragment to actin is shown. In three of the four procedures used, sedimentation, light scattering, and inhibition of actin-activated ATPase, the actin binding of the soluble 23-kDa fragment was observed, while in the fourth actin was shown to bind to immobilized 23 kDa on the nitrocellulose membrane. This latter procedure was also observed recently by Cheung and Reisler (1988). The actin binding of the 23-kDa fragment was quite unexpected, since there were no indications from the rather extensive EDC-mediated cross-linking studies (Mornet et al., 1981; Sutoh, 1983; Chen et al., 1985) that the 23-kDa domain participates in the formation of the actin–S-1 interface. However, the lack of cross-link formation between EDC-activated carboxyl groups and the ϵ-amino groups of lysines does not necessarily indicate the lack of proximity between specific regions of S-1 and actin. This was specifically shown in the case of a region stretching between SH_2 and SH_1 thiols on the 20-kDa domain, which has been implicated in actin binding by various methods (Katoh & Morita, 1984; Katoh et al., 1985; Chaussepied et al., 1986b) but not by cross-link formation. Moreover, there are other results which indirectly suggest proximity of the 23-kDa domain to the actin–S-1 interface. These include studies showing that bound F-actin inhibits the papain or thermolysin cleavage at the 23-kDa/50-kDa junction (Applegate & Reisler, 1983), modifies the trypsin cut in the presence of nucleotides at this junction (Muhlrad & Hozumi, 1982), and also modifies the cut near the N-terminus of 23 kDa (Hozumi, 1983). Finally, two 23-kDa functionalities, the N-terminus and the ATP binding site, whose locations were defined in the EM image of myosin (Winkelmann et al., 1986; Sutoh et al., 1986), are near the actin–myosin interface (Tokunaga et al., 1987). All these findings make reasonable the assumption that an actin binding site is located on the 23-kDa fragment. The binding of the 23-kDa fragment to actin is probably electrostatic in nature as indicated by the dissociation of the 23-kDa–actin complex with increasing ionic strength. The actin overlay of peptides, performed after a partial NCS cleavage of the 23-kDa fragment, showed an actin binding site on a peptide containing the 130–204 stretch of the fragment. This peptide contains a positively charged cluster of residues [Arg-143 to Arg-147 (Tong & Elzinga, 1983)]. This cluster might be responsible for the actin binding by the 23-kDa fragment which probably involves an electrostatic interaction. The 23-kDa fragment, as indicated by the light-scattering measurements, has a relatively high affinity for actin ($K_{assoc} = 1.25 \times 10^7$ M^{-1}) which is about $^1/_{50}$th of the affinity of S-1 to actin under similar conditions (Greene & Eisenberg, 1980). The isolated fragment was found to inhibit the actin-activated ATPase activity of S-1, which may indicate that the fragment prevents the proper binding of S-1 to actin by partially occupying its myosin binding site. These findings—the high affinity and specificity of binding—argue against the possibility that the actin binding of the isolated 23-kDa fragment is artifactual rather than authentic. Another question is whether the actin binding site on the 23-kDa fragment is operational under physiological conditions; i.e., it constitutes a part of the actin–myosin interface during the cross-bridge cycle. Further studies are needed to answer unambiguously this question because the lack of direct evidence about the participation of the 23-kDa fragment in forming the actin binding site in native S-1.

The actin binding of the 23-kDa fragment is ATP dependent: ATP, and less effectively PP_i and ADP, dissociates the actin–23-kDa complex. The dissociating effect is quite specific and is not due to increased ionic strength upon addition of ATP and ATP analogues. The dissociating effect of ATP upon actin–23 kDa differs from that upon the acto–S-1 complex in two respects: (1) Mg promotes the dissociation of acto–S-1 but inhibits that of the actin–23-kDa complex; (2) ATP dissociates acto–S-1 in micromolar concentration (Stein et al., 1979) while actin–23 kDa in millimolar concentration. Similar findings were obtained by Chaussepied et al. (1986c), who isolated a 30-kDa fragment consisting of the 20-kDa fragment and the C-terminal 10-kDa peptide of the 50-kDa domain. The 30-kDa fragment also binds actin in an ATP-dependent manner that is inhibited by Mg. It is well-known that Mg greatly contributes to the tight binding of ATP to myosin (Lymn & Taylor, 1970; Kelemen & Muhlrad, 1971), and one may assume that a prerequisite for this Mg effect is the binding of Mg (in complex with ATP) to a specific site on S-1. The lack of the stabilizing effect of Mg on ATP binding in the case of the N-terminal 23-kDa and the C-terminal 30-kDa fragments may indicate that the Mg binding site does not reside on these regions of S-1 [as previously proposed by Chaussepied et al. (1986c)]. This means that the binding site is located either on a 40-kDa stretch of the central 50-kDa domain of the heavy chain (starting from the N-terminus of the domain) or on the alkali light chain, as was suggested by Matsumoto and Morita (1983).

The fact that two nonoverlapping S-1 fragments, 30 kDa (Chaussepied et al., 1986c) and 23 kDa, bind to actin in an ATP- or a PP_i-dependent manner may indicate that there are two (poly)phosphate binding sites on S-1. This possibility is supported by sequence homologies with other mononucleotide binding proteins. According to Walker et al. (1982) and Moller and Amons (1985), the sequence GXXXXGK is characteristic for the phosphate binding site of these proteins, and this sequence appears twice in S-1, as 178–184 and 632–638 on the 23-kDa and at the 50-kDa/20-kDa junction on the 30-kDa fragment, respectively. The phosphate binding function of the 178–184 site on the 23-kDa fragment is supported by the fact that it is located in the middle of the "consensus" ATP binding site [residues 175–194 (Atkinson et al., 1986)], where the Ser-180 residue resides [shown to be specifically modified by near-UV irradiation in the presence of MgADP and vanadate (Grammer et al., 1988)].

The present preparation of the soluble 23-kDa fragment completes the isolation and renaturation of all the tryptic-resistant fragments of the S-1 heavy chain. All of these have ordered secondary structure and retain some function. All three fragments bind to F-actin; the binding of the 20- and 23-kDa fragments is tight, while the binding of the 50-kDa fragment is weak (Muhlrad & Morales, 1984; Muhlrad et al., 1986). The 20-kDa fragment also binds to light chains (Chaussepied et al., 1986b,c; Ueno et al., 1985). The 23-kDa fragment binds actin in an ATP-dependent manner. All these studies point to the usefulness of the isolated soluble S-1 fragments in the investigation of the structural basis of myosin function.

ACKNOWLEDGMENTS

I thank Prof. M. F. Morales and Dr. P. Chaussepied for

stimulating discussions and for critical reading of the manuscript and Prof. J. T. Yang, Dr. S. Kubota, and Dr. C.-S. C. Wu for use of their spectropolarimeter and for helping to interpret the CD spectra. The excellent technical help of Y. M. Peyser and N. Chaussepied is gratefully acknowledged.

Registry No. ATP, 56-65-5; ADP, 58-64-0; L-Trp, 73-22-3; PP_i, 14000-31-8; ATPase, 9000-83-3.

References

Applegate, D., & Reisler, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7109–7112.

Ando, T., Duke, J. A., Tonomura, Y., & Morales, M. F. (1982) *Biochem. Biophys. Res. Commun. 109*, 1–6.

Atkinson, M. A. L., Robinson, E. A., Appellla, E., & Korn, E. D. (1986) *J. Biol. Chem. 261*, 1844–1848.

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

Burke, M., & Sivaramakrishnan, M. (1986) *J. Biol. Chem. 261*, 12330–12336.

Burke, M., Zaager, S., & Bliss, J. (1987) *Biochemistry 26*, 1492–1496.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Chang, C. T., Wu, C.-S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Chaussepied, P., Mornet, D., Audemard, E., Derancourt, J., & Kassab, R. (1986a) *Biochemistry 25*, 1134–1140.

Chaussepied, P., Mornet, D., Audemard, E., Kassab, R., Goodearl, A. J., Levine, B. A., & Trayer, I. P. (1986b) *Biochemistry 25*, 4540–4547.

Chaussepied, P., Mornet, D., & Kassab, R. (1986c) *Biochemistry 25*, 6426–6432.

Chen, T., Applegate, D., & Reisler, E. (1985) *Biochemistry 24*, 137–144.

Cheung, P., & Reisler, E. (1988) *Arch. Biochem. Biophys. 265*, 272–278.

Chou, P. Y., & Fasman, C. D. (1974) *Biochemistry 13*, 222–245.

Grammer, J. C., Cremo, C. R., & Yount, R. G. (1988) *Biophys. J. 53*, 237a.

Greene, L. E., & Eisenberg, E. (1980) *J. Biol. Chem. 255*, 543–548.

Hozumi, T. (1983) *Biochemistry 22*, 799–804.

Katoh, T., & Morita, F. (1984) *J. Biochem. 96*, 1223–1230.

Katoh, T., Katoh, H., & Morita, F. (1985) *J. Biol. Chem. 260*, 6723–6727.

Kelemen, G. Sz., & Muhlrad, A. (1971) *Biochim. Biophys. Acta 235*, 503–510.

Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.

Lymn, R. W., & Taylor, E. W. (1970) *Biochemistry 9*, 2975–2983.

Mahmood, R., & Yount, R. G. (1984) *J. Biol. Chem. 259*, 12956–12959.

Matsumoto, A., & Morita, F. (1983) *J. Biochem. 93*, 943–953.

Moller, W., & Amons, R. (1985) *FEBS Lett. 186*, 1–7.

Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature 292*, 301–306.

Mornet, D., Ue, K., & Morales, M. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 736–739.

Muhlrad, A. (1988) *Biophys. J. 53*, 238a.

Muhlrad, A., & Hozumi, T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 958–962.

Muhlrad, A., & Morales, M. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1003–1007.

Muhlrad, A., & Kessel, M. (1987) *J. Muscle Res. Cell Motil. 8*, 65.

Muhlrad, A., Kasprzak, A. A., Ue, K., Ajtai, K., & Brughardt, T. P. (1986) *Biochim. Biophys. Acta 869*, 128–140.

Okamoto, Y., & Yount, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1575–1579.

Rodney, A. J., & Doolittle, R. F. (1985) *Biochemistry 24*, 162–170.

Setton, A., & Muhlrad, A. (1984) *Arch. Biochem. Biophys. 235*, 411–417.

Setton, A., Dan-Goor, M., & Muhlrad, A. (1988) *Biochemistry 27*, 792–796.

Shechter, Y., Patchornik, A., & Burstein, Y. (1976) *Biochemistry 15*, 5071–5075.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.

Stein, L., Schwartz, R. P., Chock, P. B., & Eisenberg, E. (1979) *Biochemistry 18*, 3895–3909.

Sutoh, K. (1983) *Biochemistry 22*, 1579–1585.

Sutoh, K., Yamamoto, K., & Wakabayashi, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 212–216.

Suzuki, R., Nishi, N., Tokura, S., & Morita, F. (1987) *J. Biol. Chem. 262*, 11410–11412.

Szilagyi, L., Balint, M., Sreter, F. A., & Gergely, J. (1979) *Biochem. Biophys. Res. Commun. 87*, 936–945.

Tokunaga, M., Sutoh, K., Toyoshima, C., & Wakabayashi, T. (1987) *Nature (London) 329*, 635–638.

Tong, S. W., & Elzinga, M. (1983) *J. Biol. Chem. 258*, 13100–13110.

Tonomura, Y., Appel, P., & Morales, M. F. (1966) *Biochemistry 5*, 515–521.

Torgerson, P. (1984) *Biochemistry 23*, 3002–3007.

Ueno, H., Katoh, T., & Morita, F. (1985) *J. Biochem. (Tokyo) 97*, 1785–1793.

Walker, J. E., Saraste, M., Runswick, M. J., & Gay, N. J. (1982) *EMBO J. 1*, 945–951.

Weeds, A. G., & Taylor, R. S. (1975) *Nature 257*, 54–56.

Werber, M. M., Peyser, Y. M., & Muhlrad, A. (1987) *Biochemistry 26*, 2903–2909.

Winkelmann, D. A., & Lowey, S. (1986) *J. Mol. Biol. 188*, 595–612.