Effect of Mild Heat Treatment on Actin and Nucleotide Binding of Myosin Subfragment 1†

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Received May 21, 1987; Revised Manuscript Received August 11, 1987

ABSTRACT: Chymotryptic subfragment 1 (S-1) prepared from rabbit skeletal myosin has lost its ATPase activity upon incubation at 35 °C for 3 h. The loss in ATPase activity was accompanied by the perturbation of the structure of the 50K domain as indicated by a dramatic increase in the tryptic susceptibility of this domain without any change in the susceptibility of the other domains of S-1. The perturbation starts at the C-terminal region of the domain as suggested by the appearance of a 29K intermediate protein band in the tryptic peptide pattern of the heat-treated S-1. The heat-treated molecule essentially retained its actin and polyphosphate binding ability, and the actin binding was still sensitive to the presence of ATP or pyrophosphate. However, as opposed to native S-1, in heat-treated S-1 the addition of ATP does not induce an increase in tryptophan fluorescence, and, in the case of the treated species, the fluorescence of 1,N^6-ethenoadenosine 5′-diphosphate added to the mixture is quenchable by acrylamide. This latter observation suggests that the binding of the adenine ring of the nucleotide has been altered following the heat treatment. The results indicate that the actin and polyphosphate binding sites of S-1 are distinct and that they are relatively independent of the adenine ring binding site.

The interaction between myosin, actin, and ATP is the molecular basis of contraction. A well-defined segment of myosin, called chymotryptic subfragment 1 (S-1),[1] contains separate sites responsible for ATP and actin binding (Barany & Barany, 1959). A change in occupancy at one site induces localized distortions in the molecular structure, which affect the second site. Via these distortions, a system of communication exists between the two sites, which is the basis of the function of myosin (Botts et al., 1984). Balint et al. (1978) showed that trypsin cleaves the heavy chain of S-1 into three fragments, denoted "27K", "50K", and "20K", which are aligned in this order in the primary sequence. Applegate and Reisler (1983) and Mornet et al. (1984) found that a number of proteases cleave S-1 essentially into the same fragments, indicating that these might be domains within the structure of the native S-1. The findings that the 20K and 50K fragments can be isolated in partially renatured form (Muhlrad & Morales, 1984; Muhlrad et al., 1986; Chaussepied et al., 1986b) substantially support the existence of the domain structure in S-1. It was indicated by tryptic digestion of S-1 in the presence of actin and by cross-linking experiments (Mornet et al., 1979, 1981; Sutoh, 1983; Botts et al., 1982) that the actin binding site may reside between the 20K and 50K domains; however, the involvement of the 27K domain cannot be excluded (Muhlrad & Kessel, 1986). Both 27K and 50K domains are involved probably in the formation of the ATP binding site (Szilagyi et al., 1979; Mahmood & Yount, 1984; Okamoto & Yount, 1985; Chaussepied et al., 1986c).

Setton and Muhlrad (1984), and most recently Burke et al. (1987), found that S-1, which had been incubated at 35 °C and then cooled down, essentially lost its ATPase activity. The loss of ATPase activity was accompanied by an increased tryptic susceptibility only of the 50K domain of the myosin head, implying a role of this domain in the ATPase activity of S-1. These findings also indicate that the folding and unfolding of the 50K domain of S-1 take place independently of the other domains, since the tryptic susceptibility of the latter was not affected by the mild heat treatment. Since the 50K domain was also assumed to be involved in the binding of actin, it seemed of interest to study how mild heat treatment affects the actin binding of S-1 and the regulation of this binding by nucleotides. In this paper, we show that thermal treatment induces structural changes, which lead to the loss of ATPase activity and to a profound change in the binding of the adenine ring of ATP to S-1 but do not abolish the binding of actin or of the polyphosphate moiety of the nucleotide to S-1.

Materials and Methods

Chemicals. TPCK–trypsin and α-chymotrypsin were from Millipore Corp. ε-ADP, ATP, and PMSF were from Sigma. The immunochemicals were from Amersham.

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). The digestion was terminated by adding 1 mM PMSF. S-1 was purified further by filtering on a Sephadex G-100 column, which was equilibrated with 30 mM NaCl and 10 mM Tris-HCl, pH 7.7; 0.1 mM PMSF was also present in order to inhibit any remaining proteolytic activity. Protein concentrations were obtained by absorbance, using an $E(1\%)$ at 280 nm of 5.5 and 7.5 for myosin and S-1, respectively, and an $E(1\%)$ at 290 nm for actin of 6.3. Molecular masses were

†This research was supported by Grant 00241/85 from the U.S.–Israel Binational Research Foundation and by the Muscular Dystrophy Association.
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[1] Abbreviations: S-1, chymotryptic subfragment 1; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; ε-ADP, 1,N^6-ethenoadenosine 5′-diphosphate; P_i, inorganic phosphate; PP_i, pyrophosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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assumed to be 115 and 42 kDa for S-1 and actin, respectively. Protein concentration was measured also according to Bradford (1976).

Heat Treatment. Samples of 1.6–2.0 mg/mL (14–17.5 μM) S-1 in 0.2 M sucrose, 30 mM NaCl, and 10 mM Tris-HCl, pH 7.7, were incubated at 35 °C in a water bath for various time intervals from 30 min to 3 h. Following heat treatment, the aliquot was immediately cooled in ice and then centrifuged at 130000*g* at 4 °C for 2 h in order to remove aggregated material.

Tryptic Digestion. S-1 was digested with 1:100 (w/w) TPCK–trypsin in 30 mM NaCl/10 mM Tris-HCl, pH 7.7, at 25 °C for 5–60 min. Digestion was terminated by incubating the samples in a boiling water bath for 5 min in the presence of protein sample buffer consisting of 80 mM Tris-HCl, pH 6.8, 8% glycerol, 2.4% SDS, and 1% β-mercaptoethanol.

SDS–PAGE and Immunoblot. Electrophoretic analysis of the samples was performed on 5–18% polyacrylamide gradient slab gels (Mornet et al., 1981). The gels were stained with Coomassie blue and scanned with a Helena Quick Scan densitometer. Molecular weights of the protein bands were estimated by comparing their electrophoretic mobilities with those of authentic marker proteins. In several cases, protein bands were electrophoretically transferred from the polyacrylamide gel to a nitrocellulose membrane by a Western blot technique (Burnette, 1981). The nitrocellulose was cut into two, following the transfer, and one part was stained by amido black, while the other part was immunostained using 4A 1025 anti-myosin[2] as the first antibody. The second antibody was biotinylated anti-mouse IgG. Finally, the nitrocellulose was treated with avidin and biotinylated horseradish peroxidase, and the reacting protein bands were visualized with 4-chloro-1-naphthol peroxidase substrate.

ATPase Assay. ATPase activities (micromoles of P_i per milligram of S-1 per minute) were calculated from inorganic phosphate (P_i) production (Fiske–Subarrow method). The reaction was carried out at 25 °C in 1-mL aliquots taken at various time intervals. K^+-EDTA-activated ATPase was measured in samples containing 0.22 μM S-1, 2 mM ATP, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0, and 600 mM KCl. Actin-activated ATPase was measured in samples containing 0.1 μM S-1, 0.5 μM F-actin, 1 mM ATP, 2 mM $MgCl_2$, and 20 mM imidazole hydrochloride, pH 7.0. Incubation time was chosen so that not more than 15% of the ATP was hydrolyzed.

Actin Binding Assay. To 2 μM S-1 in 50 mM NaCl, 1 mM $MgCl_2$, and 5 mM sodium phosphate buffer, pH 7.0, F-actin was added in various concentrations from 2 to 45 μM, and the mixtures were then centrifuged at 100000*g* for 1 h at 4 or 20 °C (Chalovitch & Eisenberg, 1982). The experiment was carried out either in the presence or in the absence of 4 mM MgATP or 2 mM $MgPP_i$. In the presence of MgATP or $MgPP_i$, the concentration of S-1 was 4 μM. The supernatant was separated from the pellet and measured for protein concentration, and samples were prepared for SDS–PAGE. The pellet was dissolved in protein sample buffer (see Tryptic Digestion), and both supernatant and pellet were electrophoresed in a SDS–PAGE system, as described above. The Coomassie blue stained slab gels were quantitatively evaluated by densitometry. Appropriate S-1 controls without F-actin, and F-actin controls without S-1, were also run. S-1 and actin contents in supernatant and pellet (free and bound species, respectively) were calculated from the above data, and affinity constants were estimated from either Scatchard or double-reciprocal plots.

Fluorescence Measurements. Fluorescence spectra were recorded in a Perkin-Elmer Model MPF 44 spectrofluorometer, using a thermostated cell holder. The tryptophan fluorescence was measured on 1.5 μM S-1 in 200 mM KCl, 2 mM $MgCl_2$, and 10 mM sodium phosphate buffer, pH 7.0 at 20 °C, according to Werber et al. (1972). The excitation wavelength was 295 nm, so as to ensure that only tryptophan residues were excited. After the emission spectrum was recorded, 1 mM ATP was added to the solution, and the spectrum was immediately recorded again. In reporting the effect of ATP on the tryptophan fluorescence, the fluorescence intensities were measured at the peak of the spectrum (334 nm), in the absence and in the presence of ATP, and the ATP-induced fluorescence increments were expressed as percentages of the fluorescence intensity obtained in the absence of ATP.

The fluorescence of ϵ-ADP was measured at 15 °C, using an excitation wavelength of 320 nm and an emission wavelength of 410 nm. The interaction of ϵ-ADP with S-1 was studied by quenching the fluorescence of the free nucleotide with acrylamide, as described by Ando et al. (1982). ϵ-ADP was added in 0.5 μM increments to 4 μM S-1 in 200 mM acrylamide, 2 mM $MgCl_2$, 5 mM KCl, and 40 mM Tris-HCl, pH 7.7, and the ϵ-ADP fluorescence was measured after each addition. In a control experiment, the fluorescence of ϵ-ADP was measured under the same conditions but in the absence of S-1. The fluorescence of the S-1 bound ϵ-ADP was estimated from the difference of the fluorescence data obtained in the presence and absence of S-1.

Pyrophosphate Binding Assay. The binding of $[^{32}P]PP_i$ to S-1 was determined by the gel filtration procedure of Hummel and Dreyer (1962). A column (0.6 × 50 cm) of Sephadex G-25 medium was equilibrated with 30 mM NaCl, 2 mM $MgCl_2$, 200 mM sucrose, 10 mM Tris-HCl, pH 7.7, and $[^{32}P]PP_i$ in various concentrations from 5×10^{-8} M to 2.5×10^{-6} M at 20 °C. Then 0.8 mL of 45 μM S-1 in the equilibration solution was placed on the column, and fractions of 1.7 mL were collected. The eluted fractions were assayed for S-1 content (OD_{280nm}) and for radioactivity. The value of moles of PP_i bound per mole of S-1 (r) was calculated from the average of $[PP_i]$ in the radioactive peak and in the trough, divided by the [S-1]. The affinity of PP_i to S-1 and the maximal number of PP_i bound to S-1 (n) were estimated from the r values and from the free $[PP_i]$ using Scatchard plots.

Results

We have recently reported (Setton & Muhlrad, 1984) that S-1 loses its K^+(EDTA) or Ca^{2+}-activated ATPase activity upon incubation at 35 °C for 2 h. At the end of the incubation period, S-1 is rather aggregated as revealed by the increased turbidity of the solution. More recently, we have carried out the heating in the presence of 0.2 M sucrose in order to reduce aggregation. During the heat treatment, aliquots of S-1 were withdrawn, cooled in ice, and centrifuged at 130000*g* at 4 °C for 2 h to remove aggregated material. The measured actin- and K^+(EDTA)-activated ATPase activities of the S-1 aliquots are in Figure 1. Both K^+(EDTA)- and actin-activated ATPase activities significantly decrease after 3 h of incubation at 35 °C, showing that the loss of ATPase activity observed in our earlier experiments was not due to the aggregation of S-1. The inactivation of both ATPase activities followed the same first-order kinetics, with a rate constant of inactivation of 0.011 min^{-1} for both K^+(EDTA)- and actin-activated AT-

[2] The 4A 1025 antibody (IgG) was developed against human skeletal myosin; its epitope was located within the 18K N-terminal region of the 50K domain of S-1 (Dan-Goor, Silberstein, Kessel, and Muhlrad, submitted for publication).

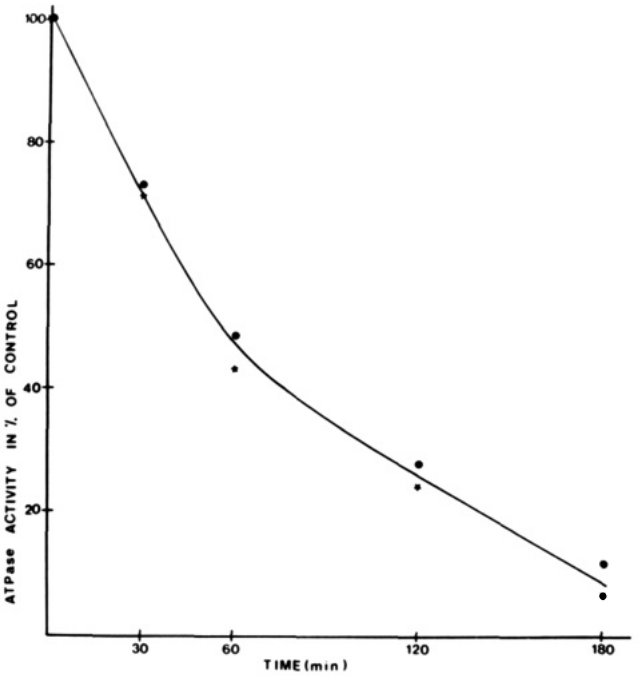

FIGURE 1: Effect of mild heat treatment on the K^+(EDTA)- and actin-activated ATPase activity of S-1. For conditions for heat treatment and ATPase activity assays, see Materials and Methods. (●) K^+(EDTA)-activated ATPase activity; (★) actin-activated ATPase activity. 100% activity is 6.35 and 1.15 μmol of P_i mg^{-1} min^{-1} for K^+(EDTA)- and actin-activated ATPase activity, respectively.

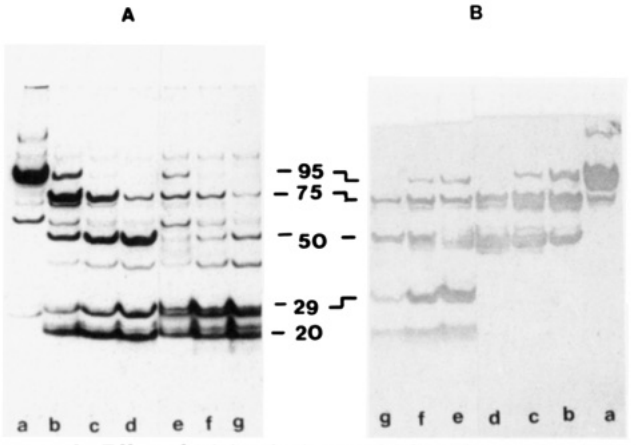

FIGURE 2: Effect of 3-h incubation at 35 °C on the tryptic peptide pattern of S-1. For conditions for heat treatment, tryptic digestion, SDS–PAGE, and immunoblot, see Materials and Methods. (A) SDS–PAGE; (B) immunoblot with anti-50K domain antibody. (a, b, c, and d) Native S-1 digested with trypsin for 0, 5, 10, and 30 min, respectively; (e, f, and g) heat-treated S-1 digested with trypsin for 5, 10, and 30 min, respectively. Vertical numbers: molecular mass in kilodaltons.

Pases. The rate of inactivation of the K^+(EDTA)-activated ATPase was somewhat slower than that observed earlier (0.015 min^{-1}) in the absence of sucrose (Setton & Muhlrad, 1984); this suggests that sucrose not only reduces aggregation but also stabilizes the S-1 structure.

The heat treatment also led to an increased tryptic susceptibility of the 50K domain (Setton & Muhlrad, 1984). This increased susceptibility was not prevented by sucrose during thermal treatment. Upon tryptic digestion of the heat-treated S-1, the 50K band, which is a major product of the trypsinolysis of the native S-1, hardly appeared on SDS–PAGE. Instead, the intensity of the 29K band was increased following a short digestion (Figure 2A). The 29K intermediate peptide obtained from the heat-treated S-1 appears to be derived from

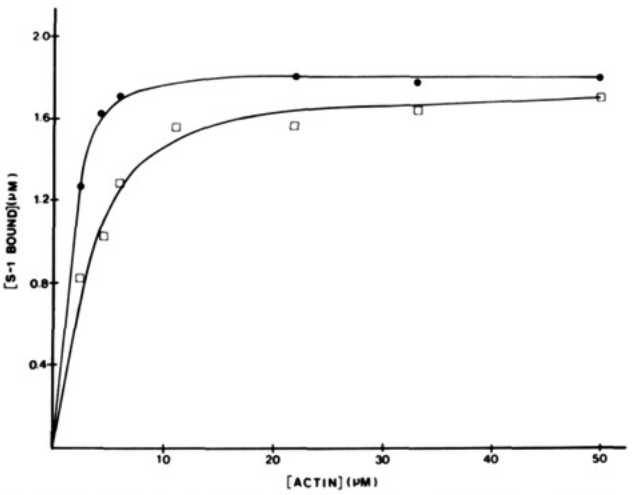

FIGURE 3: Effect of mild heat treatment on the binding of S-1 to F-actin. To 2 μM S-1, either native (●) or incubated at 35 °C for 3 h (□), F-actin was added in increasing concentrations and then centrifuged at 130000*g* for 1 h at 4 °C. The details of the actin binding assay are given under Materials and Methods.

the N-terminal portion of the 50K domain, since this peptide reacts with a monoclonal antibody (Figure 2B) whose epitope resides on the N-terminal region of the 50K domain.[2] In native, trypsin-digested S-1, the 29K band did not react with the antibody.

The effect of heat treatment on the actin binding ability of S-1 was studied by cosedimenting S-1 with increasing concentrations of F-actin in the ultracentrifuge (Figure 3). The results show that both native and heat-treated S-1 completely cosediment with F-actin, if the latter is added in a saturating concentration. The affinity and the number of S-1's bound to F-actin were estimated from the results presented in Figure 3 by using Scatchard plots. The association constants of the binding of native and heat-treated S-1 to F-actin are 1.25×10^7 and 0.93×10^6 M^{-1}, respectively. One S-1 binds per actin monomer independently of the heat treatment. The actin binding of the treated S-1 has the same ATP sensitivity as that of native S-1; i.e., 4 mM MgATP or 2 mM $MgPP_i$ dissociates both heat-treated and native S-1 from F-actin according to the results of the sedimentation experiments (results not shown). Under these conditions, the affinity of heat-treated S-1 to F-actin, as estimated from Scatchard plots, was the same as that of native S-1, 2.2×10^4 and 8.1×10^4 M^{-1} in the presence of MgATP and $MgPP_i$, respectively.

The finding that the actin binding of the heat-treated S-1 is sensitive to ATP and PP_i indicates that ATP or PP_i binds to S-1, even after S-1 has lost its ATPase activity due to thermal denaturation. Therefore, it seemed of interest to study the binding of ATP and PP_i using different approaches. One possible way to study nucleotide effects is to measure the increase in intrinsic tryptophan fluorescence of S-1 upon addition of ATP (Werber et al., 1972). We measured the effect of mild heat treatment on the ATP-induced tryptophan fluorescence increment and found that the increment decreased with the time of thermal treatment (Figure 4). The rate of loss of the ATP-induced tryptophan fluorescence increment, 0.016 min^{-1}, which followed first-order kinetics, was higher than the rate of loss in ATPase activity, 0.011 min^{-1}. A more direct method to measure nucleotide binding to S-1 was developed by Ando et al. (1982). They found that the fluorescence of free ε-ATP or ε-ADP was quenched by acrylamide but that the quenching was prevented when the ε-nucleotides were bound to S-1. Using this method, we titrated S-1, which had been subjected to heat treatment, with

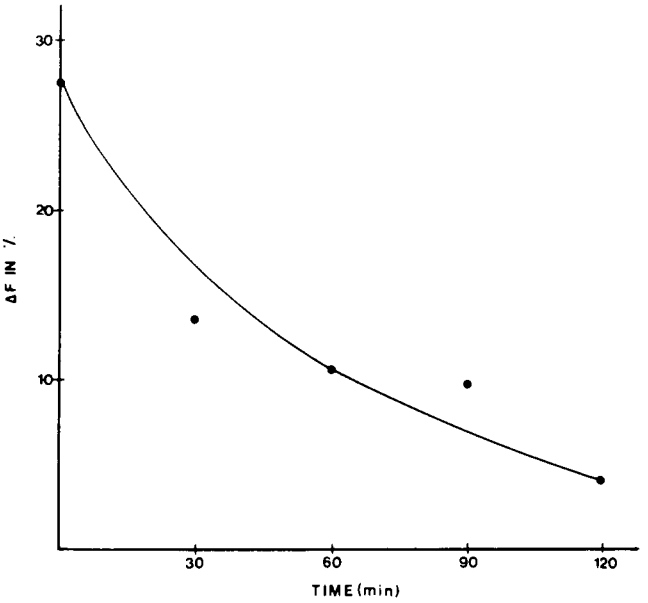

FIGURE 4: Effect of mild heat treatment of S-1 on the ATP-induced tryptophan fluorescence increment. The tryptophan emission spectrum of S-1, incubated for various time intervals at 35 °C, as indicated on the abscissa, was recorded before and after addition of 1 mM ATP, as detailed under Materials and Methods. The ATP-induced fluorescence increment at 334 nm, given on the ordinate, is expressed as the percent of fluorescence intensity recorded in the absence of ATP.

Table I: Binding of PP_i to S-1

	association constant (M^{-1})	max no. of PP_i bound to S-1 (n)
native S-1	1.5×10^6	1
S-1 incubated at 35 °C for 180 min	0.82×10^6	1

ϵ-ADP and measured the ϵ-ADP fluorescence in the presence of acrylamide (Figure 5). The amplitude of the acrylamide nonquenchable fluorescence, i.e., the amount of ϵ-ADP bound to S-1, decreased with increasing time of the thermal treatment, and the rate of decrease, which followed first-order kinetics, was identical with the rate of ATPase inactivation, 0.011 min^{-1}. The results indicate that in treated S-1 either ϵ-ADP does not bind to S-1 or the S-1 bound ϵ-ADP becomes accessible to the acrylamide quencher. Finally, in order to obtain information about the binding of the polyphosphate moiety of the nucleotide, we measured the binding of PP_i to S-1 (Table I) and found, from the analysis of Scatchard plots, that the affinity of S-1 to PP_i only moderately decreased following thermal treatment and 1 mol of pyrophosphate was bound per mole of S-1 in both the native and the heat-treated species.

Discussion

The results of this paper support the hypothesis that S-1 consists of more than one independently folded entity or domain as defined by Rossmann and Argos (1981). Mild heat treatment has been found to perturb the structure of one part of the molecule, the 50K fragment, while leaving the rest of the molecule unchanged. This finding is in agreement with our former results (Setton & Muhlrad, 1984) and those of Burke and co-workers (Burke & Sivaramakrishnan, 1986; Burke et al., 1987). Our results also indicate that the perturbation of the structure of the 50K domain may start at a region which is at 29K distance from its N-terminal, since this region becomes susceptible to tryptic attack after heat treatment as implied by the appearance of a 29K intermediate

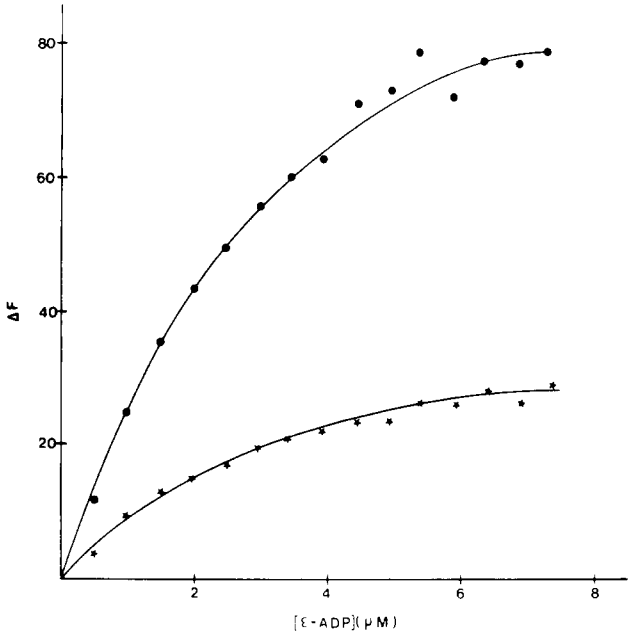

FIGURE 5: Titration of binding of ϵ-ADP to S-1 in the presence of acrylamide quencher. Increasing concentrations of ϵ-ADP were added to 4 μM S-1 in the presence of 200 mM acrylamide, and its fluorescence was recorded as described under Materials and Methods. The difference of fluorescence intensity (ΔF) in arbitrary units at 410 nm was measured in the presence and in the absence of S-1. ΔF is given on the ordinate. Native S-1 (●); S-1 incubated at 35 °C for 1 h (★).

peptide, which derived from the N-terminal segment of the 50K domain.

The loss of ATPase activity concomitant with the perturbation of the structure of the 50K domain suggests that this domain constitutes a part of the catalytic site of S-1. It was shown by Chaussepied et al. (1986a) that a thrombin cut at Lys-560 eliminates the ATPase activity of S-1. The same authors (Chaussepied et al., 1986b) observed a polyphosphate binding site on the 50K domain, and according to Mahmood and Yount (1984), the ribose moiety of a photoaffinity ATP label, 3′-*O*-(4-benzoyl)-ATP, also binds to this domain. These findings make the aforementioned possibility plausible. On the other hand, other photoaffinity labeling studies (Szilagyi et al., 1979; Okamoto & Yount, 1985; Atkinson et al., 1986) have shown that at least the purine binding site of ATP is located at the C-terminal region of the 27K domain of S-1. The foregoing indicates that at least two domains of S-1 may be involved in forming the ATP binding site. Our present results, showing that the loss of ATPase activity is *not* necessarily accompanied by the loss of PP_i or ATP binding, may indicate that the site which is responsible for the catalysis is not identical with the ATP binding site.

Although no serious damage was observed to the binding of the polyphosphate moiety of ATP, the fact that the fluorescence of ϵ-ADP added to heat-treated S-1 became quenchable by acrylamide indicates a changed interaction between the adenine ring of the nucleotide and the treated S-1. Either the nucleotide does not bind well to heat-treated S-1 or the binding is altered. According to Ando and Duke (1983), the binding of ϵ-ATP to S-1 consists of two steps: first, the phosphate moiety binds, while the adenine moiety is still on the rim of the active site and accessible to acrylamide; the adenine moiety is then pulled into a crevice which prevents the quenching of the fluorescence by acrylamide. The alteration of the above mechanism without the abolition of the binding seems plausible since after the thermal treatment ATP still binds to some extent as indicated by the surviving ATP

sensitivity of the actin binding of the heat-treated S-1. The increased acrylamide quenching of ϵ-ADP fluorescence observed following the binding of the nucleotide can be explained by structural changes that occur at the adenine ring binding site following the heat treatment. These changes seem to prevent the second step of the binding process, i.e., the tight binding of the adenine ring. The observed weakened adenine ring and the essentially unimpaired pyrophosphate binding after heat treatment indicate the existence of separate adenine ring and polyphosphate binding sites in S-1. Since the impairment of the adenine ring binding was concomitant with unfolding the 50K domain, it is suggested that the 50K domain is involved in the binding of the adenine ring.

The loss of the ATP-induced tryptophan fluorescence increment after heat treatment can be due either to the lack of ATP hydrolysis or to the impaired communication between the catalytic site and the ATP-sensitive tryptophan (Botts et al., 1984). This latter suggestion is in accordance with our previous suggestions that the ATP-sensitive tryptophan(s) of S-1 reside(s) on the 50K domain (Muhlrad et al., 1986; Werber et al., 1987).

The actin binding of S-1, either in the presence or in the absence of ATP, was not affected very much by mild heat treatment even though actin has been shown to cross-link with 50K (Mornet et al., 1981; Sutoh, 1983). This observation indicates either that the binding site on the 50K domain is normally not essential for actin binding or that the structure of this site is undamaged by mild heat treatment. This latter case is unlikely because the actin binding site is rather large according to electron microscopic evidence (Toyoshima & Wakabayashi, 1985) and such a large region would probably be affected by thermal treatment. It is also relevant that not only electrostatic (which can be probed by cross-linking) but also hydrophobic interactions on 20K and 27K domains participate in S-1–actin binding (Katoh & Morita, 1984; Muhlrad & Kessel, 1987). These results, together with the present observations, would suggest that the hydrophobic interactions predominate in the determination of the affinity between actin and myosin.

In conclusion, the present paper shows that S-1, which has lost its ATPase activity and its capacity to bind the adenine ring of nucleotide tightly, retains its actin and polyphosphate binding capability. These findings suggest that the sites responsible for actin and polyphosphate binding have a distinct structure, allowing the adenine ring binding site to be altered selectively.

Acknowledgments

We are indebted to Prof. M. F. Morales and Drs. M. M. Werber and P. Chaussepied for stimulating discussions and for critical reading of the manuscript.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3; PP_i, 14000-31-8.

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