Identification of Polyphosphate Recognition Sites Communicating with Actin Sites on the Skeletal Myosin Subfragment 1 Heavy Chain†

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ABSTRACT: Using the thrombin-cut [68–30 kilodalton (kDa)] myosin subfragment 1 (S-1) whose heavy chain has been selectively split within the central 50-kDa region, at Lys-560, with concomitant specific alterations of the ATPase and actin binding properties [Chaussepied, P., Mornet, D., Audemard, E., Derancourt, J., & Kassab, R. (1986) *Biochemistry 25*, 1134–1140; Chaussepied, P., Mornet, D., Barman, T., Travers, F., & Kassab, R. (1986) *Biochemistry 25*, 1141–1149], we have isolated and renatured the COOH-terminal 30-kDa fragment associated with the alkali light chains by the procedure recently described [Chaussepied, P., Mornet, D., Audemard, E., Kassab, R., Goodearl, J., Levine, B., & Trayer, I. P. (1986) *Biochemistry 25*, 4540–4547]. The 30-kDa peptide preparation was found to exhibit a crucial feature of the native S-1; namely, it interacts with F-actin in an adenosine 5′-triphosphate (ATP)-dependent manner. Studies by ultracentrifugation, turbidity measurements, and chemical cross-linking experiments showed that the acto–30-kDa peptide complex was dissociated almost completely by the γ-phosphoryl group containing ligands ATP, 5′-adenylyl imidodiphosphate, and pyrophosphate, to a lesser extent by ADP, and not at all by AMP and inorganic phosphate. The maximal dissociating effect is operating with the thrombic 30-kDa entity, whereas the 22-kDa fragment produced by staphylococcal protease is only slightly dissociated. In contrast, the tryptic 20-kDa fragment binds irreversibly to actin. The results imply that the release of actin from its strong recognition sites on the 20-kDa region requires mainly a transmitted, favorable interaction between the phosphoryl compounds tested and the 10-kDa difference segment lying at the NH_2-terminus of the 30-kDa element; this segment includes residues 561–640 corresponding to the COOH-terminal primary structure of the tryptic 50-kDa domain and the 50–20-kDa connector segment. This conclusion is consistent with the observation that the addition of the dissociating ligands to the 30-kDa peptide results in a 30% quenching of the fluorescence of the unique tryptophan residue present at position 594 in the NH_2-terminal part of the peptide. Finally, fluorescence quenching studies using acrylamide and the analogue 1,N^6-ethenoadenosine 5′-triphosphate did not reveal any interaction between the adenine ring and the 30-kDa peptide, suggesting that the dissociation of actin is consequent only on the attachment of the polyphosphate chain to the 30-kDa fragment. Because this portion of the myosin head appears to contain intercommunicating actin and phosphoryl group subsites, it would play in vivo a major role in the cyclic interaction of the cross-bridges with the thin filament. Unlike the 20-kDa peptide, the isolated 30-kDa preparation is a valuable material for the study of the molecular mechanisms of nucleotide-modulated actin dissociation from the S-1 heavy chain.

Muscle contraction takes place through the cyclic interaction of adenosine 5′-triphosphate (ATP)[1] and actin with the cross-bridges representing the globular heads (S-1) of myosin (Huxley, 1957). During the ATPase cycle of myosin, the binding of ATP has two major roles. First, it strongly weakens the affinity of the cross-bridges to actin and causes the dissociation of the actomyosin complex (Finlayson et al., 1969); on the other hand, the subsequent chemical conversion of ATP into ADP and P_i permits the release of the nucleotide without loss of free energy (Highsmith & Jardetsky, 1980, 1983; Eisenberg & Hill, 1985). Recent kinetic studies on acto–S-1 (Biosca et al., 1984) showed that the actomyosin dissociation occurs before the tight binding of ATP to S-1 which accompanies the isomerization of the head into an essentially irreversible M*–ATP complex (Bagshaw & Trentham, 1973). The nucleotide and actin recognition sites are known to be separate structural entities (Barany & Barany, 1959) located on the S-1 heavy chain (Wagner & Giniger, 1981; Sivaranakrishnan & Burke, 1982). However, a spatial coupling between them is thought to occur via an intersite communication system which permits the well-known close relationship between the affinities of nucleotides and actin for the myosin head and a tight linkage between the enzymatic events at the ATPase site and conformational changes at the actin–heavy-chain interface (Botts et al., 1984; Geeves et al., 1984). Understanding the molecular mechanism of the actin–myosin–ATP interaction first requires an understanding of how the ATP and actin sites are structurally interrelated on the S-1 heavy chain.

Chemical cross-linking of acto–S-1 (Mornet et al., 1981a; Sutoh, 1983) and direct binding studies between actin and peptides deriving from the COOH-terminal 20-kDa region (Katoh et al., 1985) revealed two classes of actin binding

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[1] Abbreviations: S-1, myosin subfragment 1; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; kDa, kilodalton(s); MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PP_i, pyrophosphate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMPPNP, 5′-adenylyl imidodiphosphate; ε-ATP, 1,N^6-ethenoadenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; Trp, tryptophan; LC_1, alkali light chain 1; LC_3, alkali light chain 3; LC_1' and LC_1'', degraded alkali light chain 1; EDTA, ethylenediaminetetraacetic acid.

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regions on the S-1 heavy chain. One set of sites is on either side of the connector segment joining the central 50-kDa region to the 20-kDa element (Sutoh, 1983). They would be operant essentially in the active or weakly bound states of acto–S-1 (Katoh & Morita, 1984; Botts et al., 1982); they are, indeed, cross-linkable to actin both in the absence and in the presence of nucleotides (Chen et al., 1985; personnal observations). The second set of actin sites is thought to be located in the vicinity of the SH_2-SH_1-reactive thiols on the 20-kDa segment (Cys-695 and Cys-705, respectively) and would be operant in the rigor or strongly bound states of acto–S-1 (Katoh & Morita, 1984; Katoh et al., 1985). On the other hand, our knowledge of the structural features of the S-1 ATPase site is confined to the contribution of the NH_2-terminal 27-kDa heavy-chain segment in the attachment of the adenosine moiety of the nucleotide (Szilagyi et al., 1979; Okamoto & Yount, 1985; Walker et al., 1982). However, the use of a new class of arylazido-ATP analogues led recently to the labeling of the adjacent 50-kDa region at serine-324, suggesting that the ATPase site is made up of different parts of the S-1 heavy chain (Mahmood & Yount, 1984; Okamoto & Yount, 1985). In contrast, the nature and location of the heavy-chain elements intervening in the specific binding of the polyphosphate chain of ATP are as yet totally unknown in spite of the well-established interactivity within S-1 between the actin sites and the phosphate subsites, especially the β- and γ-phosphoryl group subsites (Highsmith, 1976).

In the present work, we describe for the first time the identification on the COOH-terminal part of the 50-kDa heavy-chain domain of a region containing polyphosphate recognition sites which exhibit substrate specificity toward ATP, ADP, AMPPNP, and PP_i, and which communicate with the actin sites on the adjacent COOH-terminal 20-kDa domain of the heavy chain. Our approach is based on the use of the isolated, renatured COOH-terminal 30-kDa heavy-chain fragment of thrombin-cut (68 kDa–30 kDa)-S-1 (Chaussepied et al., 1986a). The 30-kDa entity contains intact peptides structures related to the two classes of actin sites. The results illustrate the direct binding of these ligands to the 30-kDa peptide–actin complex only through their polyphosphate chain, with a concomitant decrease of the actin affinity for the peptide, the magnitude of the actin detachment depending on the nature of the ligand in a manner quite similar to that known for acto–S-1. The nucleotide effects which are absent with the shorter tryptic 20-kDa fragment require the 561–640 residues deriving from the 50-kDa region, located at the NH_2-terminus of the 30-kDa peptide. Thus, in this heavy-chain segment of S-1 resides at least a part of the essential structural components intervening in the communication path between the actin and the polyphosphate subsites of the myosin ATPase.

Materials and Methods

Chemicals. Ultrapure guanidinium chloride was supplied by Mann Research. α-Chymotrypsin, *Staphylococcus aureus* V_8 protease, and trypsin treated with L-(tosylamido)-2-phenylethyl chloromethyl ketone were purchased from Worthington Biochemical Corp. All other chemicals were of the highest analytical grade.

Protein Preparations. S-1 (A1 + A2) from rabbit skeletal muscle myosin was isolated as described by Weeds and Taylor (1975). F-Actin was prepared according to Eisenberg and Kielley (1974) and trypsin-split (27 kDa–50 kDa–20 kDa)-S-1 as described previously (Mornet et al., 1980). Split (28 kDa–48 kDa–22 kDa)-S-1 was obtained by digestion with *Staphylococcus aureus* V_8 protease as reported previously (Chaussepied et al., 1983) using a Sepharose-immobilized protease (Cavadore et al., 1985) which permits a controlled quenching of the reaction by centrifugation. Split (68 kDa–30 kDa)-S-1 was prepared by digestion with thrombin as recently described by Chaussepied et al. (1986a).

The COOH-terminal 20-, 22-, and 30-kDa fragments were isolated complexed to the alkali light chains and in a state approaching renaturation using the procedure recently described by Chaussepied et al. (1986b). During the ethanol fractionation step, exactly 4 volumes of ethanol were added for the extraction of the 20- and 22-kDa peptide–light-chain complexes, whereas only 3 volumes were employed for the 30-kDa–light-chain preparation. Prior to use, the peptide solutions were centrifuged at 170000*g*, at 4 °C, for 1 h, and all experiments were performed on 1–3-day-old preparations.

The concentrations of S-1 and actin were measured spectrophotometrically using $A^{1\%}_{280nm}$ = 7.5 and 11.0, respectively. The concentrations of the protease-split S-1's were determined by the Bradford assay (Bradford, 1976) using S-1 as the standard. The concentrations of the COOH-terminal fragments were estimated by amino acid analysis of the content in 3-methylhistidine assuming 1.0 residue/mol of peptide (Gallager & Elzinga, 1980).

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was done in 5–18% polyacrylamide slab gels as described by Kassab et al. (1981). The gels stained with Coomassie blue were scanned with a Shimadzu Model CS 930 high-resolution gel scanner equipped with a computerized integrator.

Cross-Linking Reactions. Covalent cross-linking between F-actin and the 20-kDa–, 22-kDa–, and 30-kDa peptide–light-chain complexes, in the absence and presence of ATP (5 mM), was performed essentially by the method of Mornet et al. (1981b).

Binding Experiments. The association of actin with the different COOH-terminal peptide–light-chain preparations was analyzed in the absence and presence of nucleotides, in 10 mM HEPES buffer, 5 mM KCl, and 2 mM EDTA containing 5 μM peptide and varying concentrations of actin (0–100 μM), using sedimentation in a Beckman airfuge essentially as described by Chalovich and Eisenberg (1982) and Chaussepied et al. (1986c). The fraction of unbound COOH-terminal peptide in the supernatant was estimated by densitometric measurements on $NaDodSO_4$ electrophoretic gels with the parent protease split S-1 as standard.

The binding of F-actin to the 30-kDa–light-chain complex in the absence and presence of ATP was monitored by the turbidity at 400 nm, according to Wagner and Weeds (1979). The experiments were done in the HEPES buffer at 20 °C, in a final volume of 2.0 mL, with a Turner Model 430 spectrofluorometer. The 30-kDa peptide (0.5 μM) was mixed with varying concentrations of actin (0–1.0 μM).

Spectral Procedures. Fluorescence measurements were carried out with a Hitachi Perkin-Elmer Model MPF-4 spectrofluorometer thermostated at 20 °C. The interaction of ϵ-ATP with the 30-kDa fragment–light-chain complex was studied by the quenching of fluorescence of the free nucleotide with acrylamide as described by Ando et al. (1982) and Chaussepied et al. (1986c). Comparative tryptophan fluorescence emission spectra of the 30-kDa peptide–light-chain complex were obtained with excitation at 295 nm; the maximum emission was at 345 nm.

Results

Nucleotide-Dependent Interaction of F-Actin with the Isolated Thrombic 30-kDa Heavy-Chain Fragment of S-1.

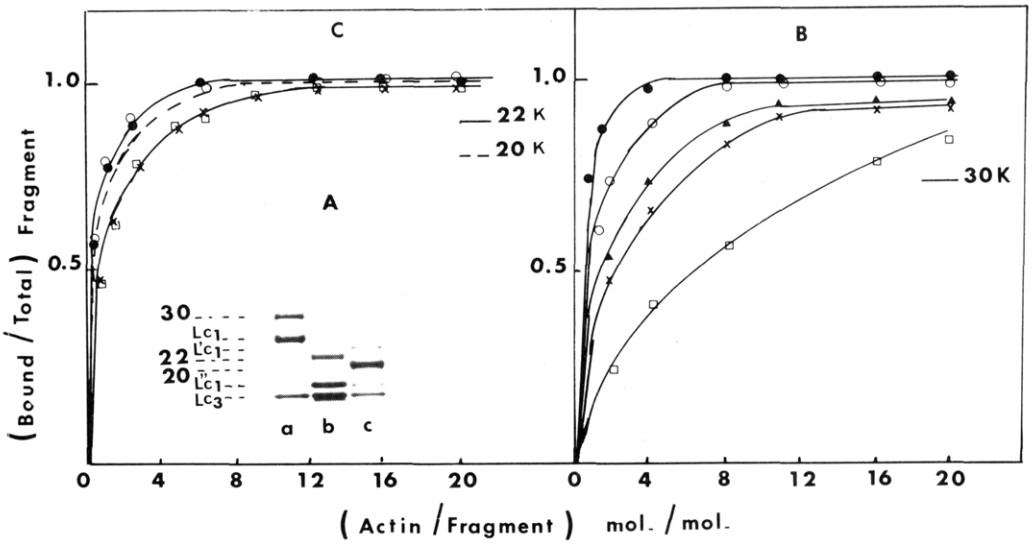

FIGURE 1: (A) Electrophoretic patterns of the isolated complexes between the alkali light chains and the COOH-terminal 30-, 22-, and 20-kDa fragments of the S-1 heavy chain. The complexes were prepared approaching renaturation by the procedure of Chaussepied et al. (1986c) using (a) thrombin-split (68 kDa–30 kDa)-S-1 (A1 + A2), (b) *Staphylococcus aureus* V_8 protease split (28 kDa–48 kDa–22 kDa)-S-1 (A1 + A2), and (c) trypsin-cut (27 kDa–50 kDa–20 kDa)-S-1 (A1 + A2). (B) Ligand-specific modulation of actin affinity for the 30-kDa peptide by ATP, AMPPNP, ADP, and PP_i. Varying concentrations of F-actin (0–100 μM) were mixed with the 30-kDa peptide preparation (5 μM) in 10 mM HEPES, 5 mM KCl, and 1 mM EDTA, pH 7.5, and were centrifuged, as indicated under Materials and Methods, in the absence (●) and presence of 5 mM ATP (□), ADP (○), AMPPNP (▲), or PP_i (×). The fraction of actin-bound fragment was estimated from the amount of free peptide in the supernatant measured as specified under Materials and Methods. (C) Influence of the phosphorylated ligands on the interaction of actin with 22- and 20-kDa peptides. Actin was cosedimented with 20-kDa peptide in the absence and presence of 5 mM ATP (---) and with 22-kDa peptide in the absence (●) and presence of 5 mM ATP (□), ADP (○), or PP_i (×) under the experimental conditions reported in Figure 1B.

The thrombin-generated COOH-terminal 30-kDa fragment which starts at Ser-561 was isolated complexed to the intact LC_1 and LC_3 light chains and in a state approaching renaturation (Figure 1A). We have also isolated by the same procedure (Chaussepied et al., 1986b) the complexes of the light chains with the shorter Gly-641–20-kDa and Glu-629–22-kDa heavy-chain segments (M. Elzinga, personal communication). The interaction of F-actin with these three different peptide preparations was analyzed in the absence and presence of nucleotides, under various experimental conditions, first using high-speed centrifugation (Figure 1B,C). At an actin:peptide molar ratio of 4, almost 100% of the 30- and 22-kDa fragments were coprecipitated with actin whereas total binding of the 20-kDa preparation was achieved at slightly higher actin:peptide ratios. Thus, the extra peptide material at the N-termini of the 30- and 22-kDa entities does not hinder, but rather favors, the binding of these fragments to actin.

The presence of millimolar concentrations of ATP, ADP, AMPPNP, or PP_i in the actin–peptide mixtures led to a dramatic change in the binding pattern of F-actin to the 30-kDa preparation as compared to the 22- and 20-kDa fragment-containing solutions. At moderate actin:peptide ratios of 2–4, ATP induced an extensive dissociation of the 30-kDa fragment from actin with the production of at least 80% unbound peptide. The two nucleotide analogues PP_i and AMPPNP were also efficient but quantitatively less than ATP in lowering the 30-kDa peptide affinity for actin. ADP also decreased the actin–30-kDa association but significantly less than the three other phosphorylated ligands. At high actin concentration, the dissociating ability of all ligands strongly decreased, suggesting the occurrence of a rapid equilibrium between 30-kDa peptide–nucleotide and acto–30-kDa peptide–nucleotide complexes. The nucleotide-mediated dissociation of the acto–30-kDa system is observable not only with the 30-kDa peptide associated with both A1 and A2 light chains but also with the 30-kDa–A2 complex (result not shown). Thus, in spite of its ability to interact with actin (Prince et al., 1981; Sutoh, 1982) and under some conditions with ATP (Winstanley et al., 1979; Burke et al., 1981), the A1 light chain does not seem to contribute significantly to this process. The association of the 20-kDa peptide with actin was unaffected by any of the phosphorylated ligands tested. For the 22-kDa peptide, however, the extent of binding to actin decreased by about 10–15% of the presence of 5 mM ATP or PP_i but not at all by ADP.

The attachment between actin and the isolated 20-kDa fragment is known to be accompanied by an increase of the actin turbidity, the magnitude of which is quite similar to that observed with the intact S-1 (Muhlrad & Morales, 1984; Katoh et al., 1985). Figure 2 illustrates the turbidimetric titration of the 30-kDa peptide with F-actin in the absence and presence of ATP. At pH 7.5, 20 °C, μ = 10 mM, the dissociation constant calculated from the first-order binding curve was 5×10^6 M^{-1}, a value close to that reported for the complexes of actin with various 20-kDa peptide preparations (Muhlrad & Morales, 1984; Katoh et al., 1985; Chaussepied et al., 1986b). The addition of millimolar concentrations of ATP at the end of the titration experiment induced an immediate abolition of the turbidity enhancement. In contrast, the nucleotide had no significant influence on the light-scattering intensity of F-actin complexed to 20- or 22-kDa peptides (data not shown).

Finally, the complexes of the three fragments with F-actin (actin:peptide ratio = 4) were cross-linked with EDC in the absence and presence of 5 mM ATP (Figure 3). The nucleotide induced the suppression of the actin–30-kDa adduct whereas the production of the cross-linked actin–20-kDa peptide and actin–22-kDa peptide together with the actin dimers and trimers was unchanged. The high extent of turbidity decrease and the almost total absence of actin–30-kDa cross-linking indicate that the nucleotide dissociation effect is exerted on both the 30-kDa–A1 and 30-kDa–A2 species.

Binding of the Nucleotide Polyphosphate Chain to the 30-kDa Fragment Monitored by the Perturbation of Tryp-

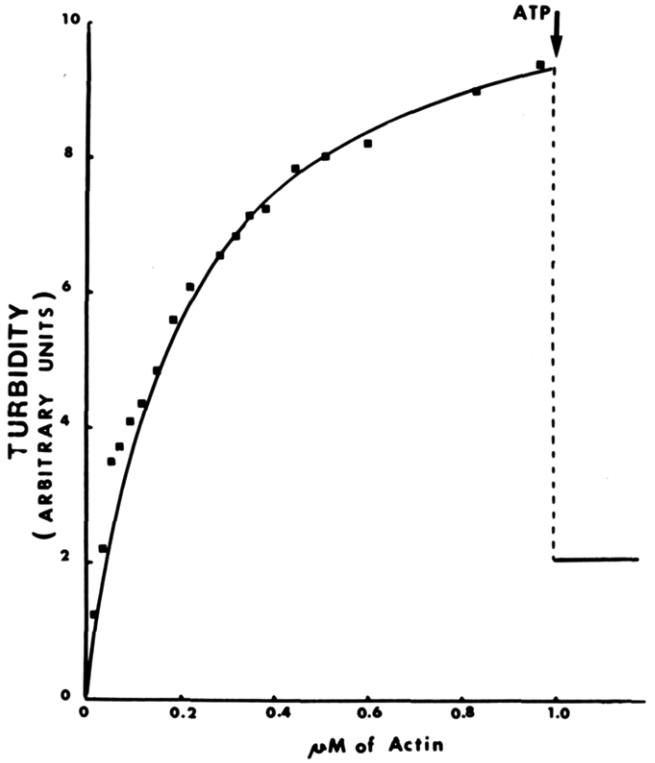

FIGURE 2: Dissociation of the actin–30-kDa peptide complex by ATP as monitored by turbidity measurements. The 30-kDa peptide (0.5 μM) was titrated with F-actin (0–1 μM) in 10 mM HEPES, 5 mM KCl, and 1 mM EDTA, pH 7.5, 20 °C. When the turbidity was close to the plateau, ATP (5 mM) was added to the protein mixture. The continuous curve was computer simulated by using K_a = [acto–30-kDa peptide]/([30-kDa peptide] − [acto–30-kDa peptide])([actin] − [acto–30-kDa peptide]). The end point lies at 11.3 as extrapolated by computer analysis. Note that this point is very near that where the ATP is added (about 9.5).

tophan-594. The nucleotide-dependent detachment of actin from the 30-kDa peptide was much more pronounced in the absence than in the presence of the divalent ions Mg^{2+} or Ca^{2+}, suggesting strong ionic interactions between the fragment and the negatively charged phosphate moieties of the ligands. Fluorescence quenching experiments with acrylamide and ϵ-ATP showed no apparent binding of the adenine ring to the peptide (data not shown), suggesting that the purine moiety is not stacking on the peptide backbone. Moreover, this polyphosphate-linked influence was not based on a local change in the ionic strength around 30 kDa brought about by the charged phosphates. Figure 4A shows clearly that an increase of the KCl concentration up to 150 mM induced the release of maximally 20% of the actin-bound 30-kDa peptide whereas 70% of the complex was dissociated by 5 mM ATP at μ = 30 mM. Finally, in Figure 4B we illustrate the direct binding of the ligand's phosphoryl groups to the 30-kDa peptide as monitored by the changes of the intrinsic fluorescence intensity of the single tryptophan present at position 594 in the N-terminal portion of the peptide [the associated alkali light chains do not contain tryptophan residues (Frank & Weeds, 1974)]. The nucleotides induced a quenching of the maximum fluorescence intensity without shifting the spectrum (Figure 4B, inset). As expected, ATP and ADP promoted the strongest (30%) and the smallest (8%) fluorescence change, respectively, whereas AMP had a very slight effect. From the first-order curve of fluorescence quenching, the apparent binding constant of ATP was 1×10^3 M^{-1}.

Discussion and Conclusions

The 30-kDa peptide–light-chain complex represents the smallest myosin derivative carrying interacting actin and phosphoryl subsites. The association of the phosphorylated ligands with the acto–30-kDa peptide exhibits some differences but several important similarities relative to acto–S-1. The apparent lack of binding of the adenine moiety is in agreement

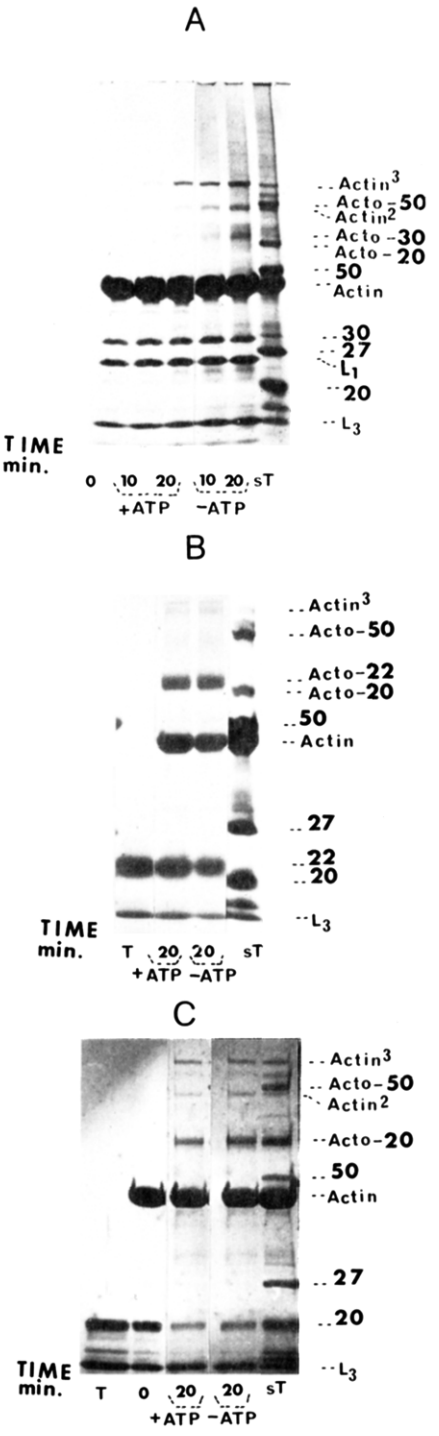

FIGURE 3: Inhibition of EDC cross-linking between actin and the 30-kDa fragment by ATP. (A) Isolated, soluble 30-kDa preparation (17 μM) was incubated at 20 °C with a 4 molar excess of F-actin and 5 mM EDC in 50 mM MES buffer, pH 6.8, in the absence and presence of 5 mM ATP. After 10- and 20-min reaction times, the mixtures were analyzed by gel electrophoresis. (B and C) The 22- and 20-kDa peptide preparations were respectively cross-linked to actin under the conditions reported in (A). The gel patterns for the 20-min reaction are shown. ST = control trypsin-split (27 kDa–50 kDa–20 kDa)-S-1 cross-linked to actin for 20 min. T = starting light-chain–22-kDa peptide or light-chain–20-kDa peptide solutions. 0 = mixture of actin and 30-kDa peptide–light-chain complex before cross-linking.

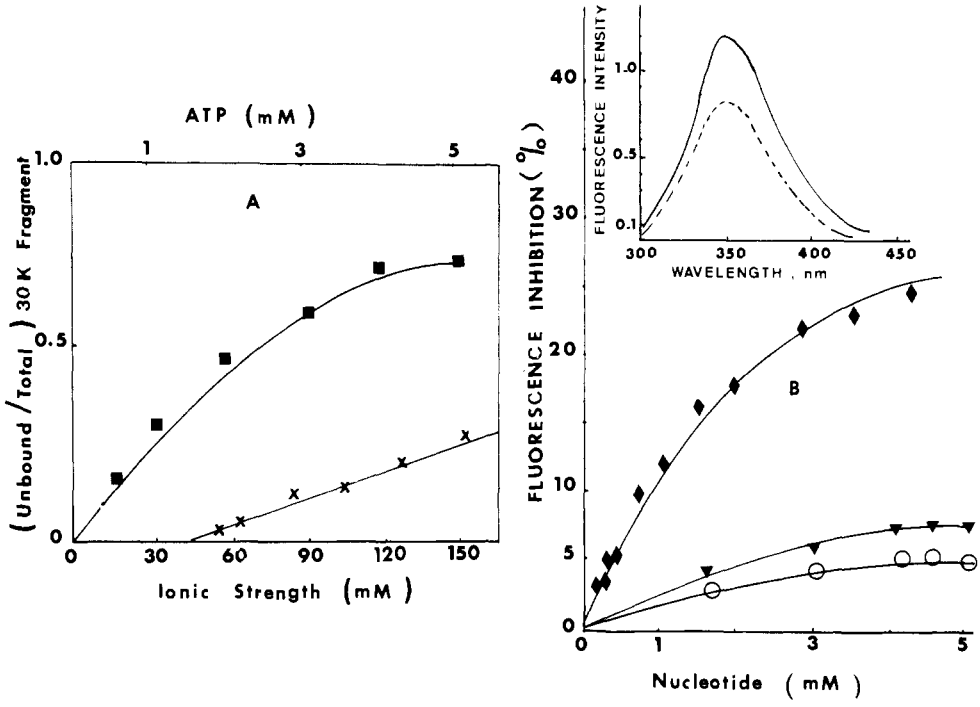

FIGURE 4: (A) Comparative influence of ATP and ionic strength on the association of actin with the 30-kDa peptide. Increasing concentrations of ATP (■) or KCl (×) were added to the mixture of 5 μM 30-kDa peptide and 10 μM F-actin. After sedimentation, the fraction of unbound peptide present in the supernatant was measured as indicated under Materials and Methods. (B) Change in the tryptophan fluorescence of the 30-kDa peptide by nucleotide binding. Increasing concentrations of ATP (♦), ADP (▼), or AMP (○) were added to the 30-kDa peptide preparation (7 μM) in 20 mM HEPES, 5 mM KCl, and 1 mM EDTA, pH 7.5, 20 °C; the percent of fluorescence quenching was measured at 345 nm, using $\% F = [(F_0 - F)/F_0] \times 100$, where F_0 = the intrinsic tryptophan fluorescence in the absence of nucleotide and F = the measured fluorescence. Inset: Fluorescence emission spectra of the 30-kDa peptide (6 μM) in the absence (—) and presence (- - -) of 5 mM ATP, pH 7.5, 20 °C.

with the assumption that the nucleoside binding subsite in S-1 resides on the N-terminal 27-kDa heavy-chain region (Szilagyi et al., 1979; Okamoto & Yount, 1985a). On the other hand, the very poor interaction of the metal–nucleotides is easily understandable, assuming that the amino acid side chains involved in the specific ligation of the divalent metal ions to S-1 (Webb et al., 1982) either are absent or are not operant in the renatured fragment. It would be possible that the 30-kDa entity represents one side of the crevice-shaped nucleotide binding site and that the other side which contains the Mg^{2+} binding site is on the 68-kDa fragment. The neutralization of the charged phosphoryl groups by the added divalent ions would prevent their favorable interaction with the 30-kDa peptide. The nonparticipation of the divalent ion in nucleotide binding could explain the relative weak affinity of uncomplexed ATP for the 30-kDa peptide. However, since the affinity of actin for the 30-kDa fragment is also weaker than that for S-1, a favorable balance between the affinities of the two ligands, actin and nucleotides, is existing on the 30-kDa material, which has permitted the experimental demonstration of the intersite communication that is actually present in the 30-kDa heavy-chain region.

The effects of the phosphorylated ligands on the acto–30-kDa peptide complex are essentially similar to those observable with acto–S-1 in the presence of Mg^{2+}–nucleotides (Taylor, 1979; Adelstein & Eisenberg, 1980). Thus, AMPPNP and PP_i were very similar and more effective than ADP in causing dissociation of the acto–30-kDa peptide complex. Substrate specificity is still mediated through the 30-kDa peptide which clearly distinguishes between the binding of ATP and ADP and propagates it to the actin sites. Since ATP caused the most extensive dissociation of the acto–30-kDa peptide as it does for acto–S-1, then this peptide must contain β- and γ-phosphoryl group subsites the occupation of which by ATP in S-1 is known to have the largest influence on actin affinity (Highsmith, 1976).

Finally, the apparent binding constant of ATP for the 30-kDa fragment (1×10^3 M^{-1}) is not different from the reported kinetic value (10^3–10^4 M^{-1}) for K1 corresponding to the M·ATP collisional complex formed during the myosin ATPase reaction (Johnson & Taylor, 1978; Chock et al., 1979; Adelstein & Eisenberg, 1980). This complex is followed by the M*·ATP intermediate which involves the tight binding of the adenosine moiety to the active site (Bagshaw & Trentham, 1973). Thus, the interaction we observed between ATP and the 30-kDa fragment would simulate the initial and very rapid anchorage of the polyphosphate chain of the nucleotide in the ATPase active site (Ando & Duke, 1983).

Although we could not directly rule out the binding of the phosphate moieties to the 20- and 22-kDa peptides without actin dissociation, the data strongly suggest the involvement of the 560–640-residue segment present on the N-terminal part of the 30-kDa peptide in phosphoryl group recognition and intersite transmission. This region includes about 75 residues which encompass the C-terminal 10-kDa portion of the central 50-kDa region and the intact 50-kDa–20-kDa connector segment, the boundaries of which are not yet clearly defined. We have surmised that the covalent union of the 20-kDa segment with the 50-kDa-deriving moiety would make the entire 30-kDa fragment, behaving as a multifunctional, two-domain-containing heavy-chain derivative. We have been encouraged in studying the mutual association of actin and nucleotides to the 30-kDa peptide, considering that interdomain interactions in S-1 could play a major role in the intersite communication. The interaction of the 30-kDa peptide with the phosphate groups rationalizes the striking influence exerted by metal-complexed and uncomplexed nucleotides on the proteolytic sensitivity of the 45–20-kDa peptide stretch in S-1

(Hozumi, 1983; Mocz et al., 1982, 1984; Applegate & Reisler, 1984; Mornet et al., 1985) which was mediated by the binding of the polyphosphate chain (Mornet et al., 1985). Because of our ignorance about the tertiary structure of the S-1 heavy chain, we cannot readily find out an unequivocal correlation between phosphoryl binding and the 30-kDa peptide structure. However, secondary structure predictions showed the critical N-terminal moiety of the 30-kDa peptide to contain several α-helices. The α-helix dipole represents, now, a well-established, crucial chemical requirement for the favorable interaction of the charged phosphates with many nucleotide binding enzymes and is thought to operate in some energy transducing systems (Hol et al., 1978; Hol, 1985).

Since the proteolytic 30-kDa peptide is obtained after various preparative steps, one can surmise that its binding to the phosphorylated ligands might occur without actin dissociation. Indeed, we know some chemically modified S-1 forms whose association with actin is not reversed by ATP binding (Pemrick & Weber, 1976). However, since this has not been observed, the 30-kDa peptide must still contain at least part of the intrinsic S-1 conformation that is competent for the intersite coupling. The latter process may be facilitated by intramolecular contacts between the specific structural features in the 50-kDa domain carrying the phosphoryl group subsites and those in the 20-kDa domain containing the actin sites. Support for such functional contacts has been recently provided by the cross-linking of these two regions with concomitant trapping of the nucleotide at the ATPase site (Chaussepied et al., 1986d).

ACKNOWLEDGMENTS

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Registry No. ATP, 56-65-5; AMPPNP, 25612-73-1; ADP, 58-64-0; PP_i, 14000-31-8.

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Effect of Temperature on the Mechanism of Actin Polymerization†

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ABSTRACT: The rate of the Mg^{2+}-induced polymerization of rabbit skeletal muscle G-actin has been measured as as function of temperature at pH 8 by using various concentrations of Mg^{2+}, Ca^{2+}, and G-actin. A polymerization mechanism similar to that proposed at this pH [Frieden, C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6513–6517] was found to fit the data from 10 to 35 °C. From the kinetic data, no evidence for actin filament fragmentation was found at any temperature. Dimer formation is the most temperature-sensitive step, with the ratio of forward and reverse rate constants changing 4 orders of magnitude from 10 to 35 °C. Over this temperature change, all other ratios of forward and reverse rate constants change 7-fold or less, and the critical concentration remains nearly constant. The reversible Mg^{2+}-induced isomerization of G-actin monomer occurs to a greater extent with increasing temperature, measured either by using *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine-labeled actin or by simulation of the full-time course of the polymerization reaction. This is partially due to Mg^{2+} binding becoming tighter, and Ca^{2+} binding becoming weaker, with increasing temperature. Elongation rates from the filament-pointed end, determined by using actin nucleated by plasma gelsolin, show a temperature dependence slightly larger than that expected for a diffusion-limited reaction.

Actin can undergo transformation from a monomeric form (G-actin) to a long helical polymer (F-actin)[1] by a polymerization process classically described as a nucleation–elongation reaction (Oosawa & Kasai, 1962). This polymerization process in vitro is strongly influenced by environmental variables such as pH, temperature, and ionic strength (Kasai et al., 1962; Kasai, 1969), and actin assembly or disassembly in vivo is probably influenced by similar factors as well as by actin binding proteins. Thus, detailed knowledge of the role such environmental factors play may be important for understanding the regulation and modulation of in vivo actin polymerization.

The temperature-dependent studies of actin polymerization to date have involved measuring the changes in either critical concentration (C_c), viscosity, or polymerization half-time (Swezey & Somero, 1982; Grazi & Trombetta, 1985; Kasai et al., 1962). Viscosity measurements, however, result in artificial filament fragmentation due to shear stresses, thereby increasing the filament number and polymerization rate. The polymerization half-time appears to be an inadequate reflection of the overall polymerization mechanism (Frieden & Goddette, 1983), while critical concentration measurements may depend upon conditions used and do not necessarily reflect only the monomer–filament equilibrium (Frieden, 1983, 1985). Thus, none of these methods has reliably characterized the full polymerization time course nor given any clues as to the mechanism.

In recent years, the use of fluorescently labeled actins has been found to yield a more sensitive and accurate measurement of actin polymerization and/or conformation [a review of these probes is given in Tait & Frieden (1983)]. Changes in the fluorescence of AEDANS-labeled actin have previously been shown to be related to a Mg^{2+}-induced isomerization of G-actin

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[1] Abbreviations: Ca–G-actin, monomeric actin with bound Ca^{2+}; Mg–G-actin, monomeric actin with bound Mg^{2+}; F-actin, polymerized filamentous actin; AEDANS-labeled actin, actin labeled with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; pyrene-labeled actin, actin labeled with *N*-(1-pyrenyl)iodoacetamide; Tris, tris(hydroxymethyl)aminomethane.

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