Binding of the Amino-Terminal Region of Myosin Alkali 1 Light Chain to Actin and Its Effect on Actin–Myosin Interaction

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ABSTRACT: The role of the amino-terminal region of myosin alkali 1 light chain (A1) in the interaction between actin and myosin subfragment-1 (S-1) was explored. Papain digestion of skeletal myosin filaments produced S-1 whose A1 was found to lose the basic 13 amino-terminal amino acid residues (A1′). We obtained three types of papain S-1 isoenzymes differing in their alkali light chain content: recombined papain S-1(A1), papain S-1(A1′), and papain S-1(A2). Both the maximum turnover rate (V_{max}) and the dissociation constant (K_m) for actin-activated papain S-1(A1′) ATPase activity were similar to those for papain S-1(A2) and remarkably larger than those for recombined papain S-1(A1). The 13 amino-terminal residue peptide of A1 (N-pep) was isolated and characterized. ^{1}H-NMR spectroscopy suggested that the N-pep was relatively immobilized in the presence of actin filaments. A cross-linking study suggested that N-pep binds to actin. The addition of N-pep to acto–S-1(A1) made K_m and V_{max} for the actin-activated ATPase activity close to those for S-1(A2). Removal of the trimethyl group from the N-pep suppressed the above effect on the actin–S-1 interaction. Our findings suggest that the amino-terminal region of A1 binds to the actin molecule to affect the mechanism of actin-activated S-1 ATPase.

Skeletal muscle myosin is composed of two heavy chains associated with two noncovalently attached light chains (Weeds & Lowey, 1971). One of the light chains has been termed the "regulatory" light chain and the other the "alkali" light chain. The alkali light chain is binding to a region of the heavy chain adjacent to the "hinge region" and may be interacting by modulating this flexible region (Burke et al., 1983; Rayment et al., 1993a). There are two distinct alkali light chains, the alkali 1 light chain (A1)[1] and the alkali 2 light chain (A2). The two alkali light chains have a common amino acid sequence except that A1 has an additional 41 residues at the amino-terminal region including a basic amino terminus and a repetitious Ala-Pro sequence (Frank & Weeds, 1974; Matsuda et al., 1981). The globular head of myosin is called subfragment-1 (S-1); it contains the ATPase site and the actin interaction site. Chymotryptic S-1 obtained from myosin filaments is separable into S-1(A1) and S-1(A2) according to alkali light chain content (Yagi & Otani, 1974). The A1 was reported to be responsible for S-1-induced actin polymerization (Chaussepied & Kasprzak, 1989). And it has been observed that S-1(A2) has a larger K_m for actin than S-1(A1) and that the V_{max} of actin-activated ATPase activity for S-1(A2) is about twice that for S-1(A1) at low ionic strength (Weeds & Taylor, 1975) and that the difference decreases as the ionic strength increases (Chalovich et al., 1984). A1 can be cross-linked to actin in its amino-terminal additional 41 residues (Sutoh, 1982), and Yamamoto observed that the cross-linking reaction decreased as the ionic strength was raised (Yamamoto & Sekine, 1983). ^{1}H-NMR studies have demonstrated a direct interaction between the additional amino-terminal region of A1 and actin; the interaction is weakened by raising the ionic strength (Henry et al., 1985; Trayer et al., 1987). Together, these data suggest that the difference in the actin-activated ATPase activities between S-1 isoenzymes arises from the interaction between actin and the additional 41 amino-terminal residues of A1. Although knowledge about the actin–A1 interaction has been required, it is still premature to draw a clear picture of how the amino-terminal region of A1 interacts with actin and of what part of the bound light chain interacts with actin.

In the present study, we show that a critical region for the actin–A1 interaction is in the 13 amino-terminal residues of A1. We show, furthermore, that this region binds with actin to modulate both the V_{max} and the K_m of the actin-activated ATPase activity of S-1(A1), using papain S-1 isoenzymes and/or the isolated 13 amino-terminal amino acid residue peptide of A1.

MATERIALS AND METHODS

HPLC Systems. Ion-exchange HPLC, gel-filtration HPLC, and reversed-phase HPLC were performed using a system composed of an ABI-1400 solvent delivery system, a 1480A injector/mixer, and a 1783A absorbance detector–controller and a system composed of a Shimadzu-SPD-6A UV spectrophotometric detector, a LC-6A liquid chromatograph, a SIL-6A auto injector, and a C-R3A chromatopac, respectively.

Preparation of Proteins. Myosin was prepared from chicken pectoralis muscle according to Perry (1955). Papain S-1 was isolated from the myosin in the presence of 2 mM EDTA according to Margossian and Lowey (1982) and then separated using ion-exchange HPLC (Figure 1a). This procedure yielded two populations of papain S-1: papain S-1(A1′) and papain S-1(A2), according to their alkali light chain content (Figure 1c). Chymotryptic S-1 isoenzymes were isolated from the myosin according to Weeds and Taylor (1975). Actin was extracted from the acetone-dried powder of chicken pectoralis

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[1] Abbreviations: A1 and A2, alkali 1 light chain and alkali 2 light chain from skeletal muscle myosin, respectively; A1′, alkali 1 light chain whose amino-terminal small peptide was removed by papain; CCD, charged coupled device; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Fmoc, 9-fluorenylmethyloxycarbonyl; HMP, *p*-(hydroxymethyl)phenoxymethylpolystyrene; LDI-TOF MS, laser-desorption ionization time of flight mass spectroscopy; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; S-1, myosin subfragment-1; TFA, trifluoroacetic acid.

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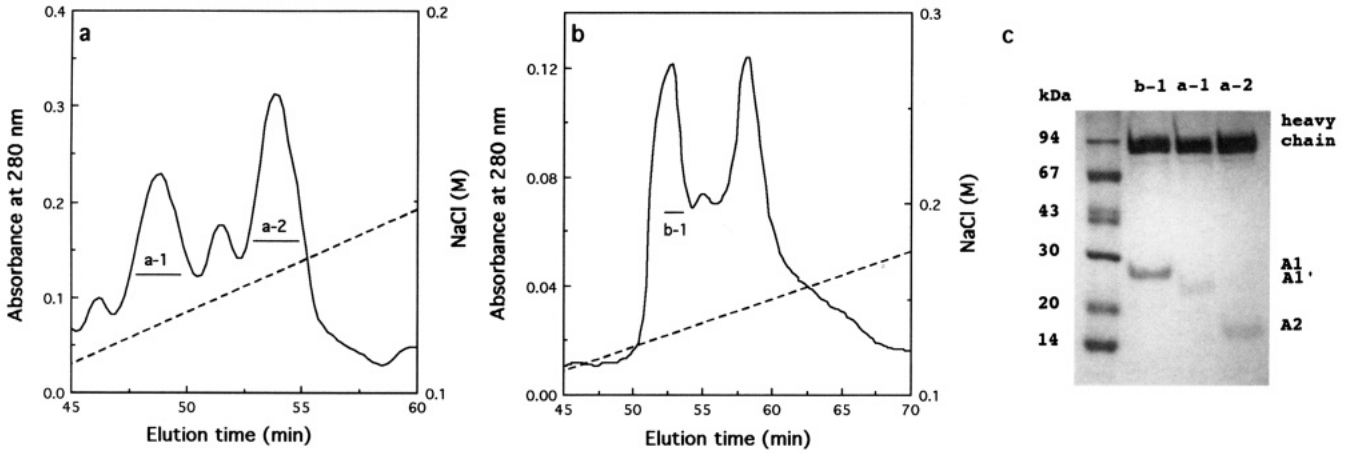

FIGURE 1: (a) DEAE-HPLC elution profile of papain S-1. Papain S-1 (2.6 mg) was applied to a Waters DEAE-8HR Protein Pak column (5 × 0.5 cm) and eluted in 20 mM Tris-HCl, pH 7.8, with a linear gradient to 1.0 M NaCl at room temperature, and the absorbance was measured at 280 nm. The flow rate was 0.4 mL/min. The fractions indicated by solid bars as a-1 and a-2 were pooled and examined with SDS–PAGE as shown in panel c. (b) Chromatographic separation of papain S-1(A1) recombined from papain S-1(A2) and A1. After the exchange process described in Materials and Methods, the protein solution (3 mL) was chromatographed as described above on a Waters DEAE-8HR Protein Pak column (5 × 0.5 cm). The fraction indicated by a solid bar as b-1 was examined with SDS–PAGE as shown in panel c. (c) Polyacrylamide gel electrophoresis on a 10% gel in the presence of SDS of the pooled fractions from the columns shown in panels a and b. Key: b-1, papain S-1(A1); a-1, papain S-1(A1′); a-2, papain S-1(A2). The first lane shows the molecular mass standards (94, 67, 43, 30, 20, and 14 kDa from the top).

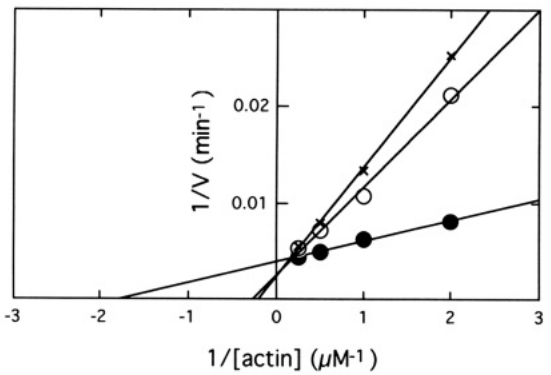

FIGURE 2: The [activity]$^{-1}$ vs [actin]$^{-1}$ plots of actin-activated ATPase activity for papain S-1(A1) (●), papain S-1(A1′) (○), and papain S-1(A2) (×). The reaction mixture contained 10 mM imidazole hydrochloride, pH 7.0, 5 mM KCl, 2 mM $MgCl_2$, 500 μM ATP, and 0.1 μM S-1 isoenzyme with 0–3 μM actin. For papain S-1(A1), K_m = 0.6 μM and V_{max} 4.3 s^{-1}. For papain S-1(A1′), K_m = 3.8 μM and V_{max} = 6.8 s^{-1}. For papain S-1(A2), K_m = 5.0 μM and V_{max} = 7.2 s^{-1}.

muscle by the method of Spudich and Watt (1971). The alkali light chains were isolated from the myosin using the method of Matsuda et al. (1981). The concentrations of the proteins were estimated by the biuret reaction using a Shimadzu-UV2100 spectrophotometer. The molecular weights (M_r) of papain S-1 heavy chain, chymotryptic S-1 heavy chain, actin, A1, A1′, and A2 were deduced from each amino acid sequence: 95 000, 92 000 (Maita et al., 1991), 42 000 (Collins & Elzinga, 1975; Vandekerckhove & Weber, 1979), 21 000 (Matsuda et al., 1981), 19 500 (Figure 3), and 16 600 (Matsuda et al., 1981), respectively. The carboxyl terminus of papain S-1 and chymotryptic S-1 has not been determined but was supposed from the tryptic fragments of papain S-1 and chymotryptic S-1 (Maita et al., 1991).

Isolation of Recombined Papain S-1(A1). A2 of papain S-1(A2) was replaced with A1 by the method used for alkali light chain exchange of chymotryptic S-1 (Wagner & Weeds, 1977). The exchange conditions were 3.2 μM papain S-1(A2), 21.4 μM A1, 2 mM EDTA, 2 mM DTT, 4.7 M NH_4Cl, and 0.1 M imidazole hydrochloride, pH 7.0. After being stirred for 30 min at 4 °C, the isolation was dialyzed overnight against

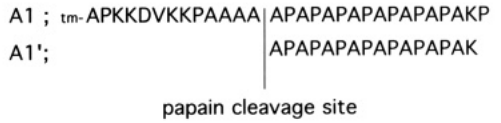

FIGURE 3: Amino-terminal sequence of A1 and A1′ obtained from chicken fast skeletal muscle myosin. The amino acid sequence of A1 has been previously described (Matsuda et al., 1981); tm-A represents N^{α}-trimethylalanine. Isolated A1′ was sequenced as described in Materials and Methods, and A-P-A-P-A-P-A-P-A-P-A-P-A-P-A-K was found. Comparison of A1 with A1′ indicated that the 13 amino-terminal amino acid residues were cut off by papain digestion of A1 light chain.

0.1 mM DTT and 20 mM Tris-HCl, pH 7.8, at 4 °C. The resulting papain S-1(A1) was then isolated using ion-exchange HPLC (Figure 1b,c).

Actin-Activated ATPase Activity Assay of S-1. The actin-activated ATPase activities of S-1 isoenzymes were calculated from the inorganic phosphate liberated, using a Shimadzu-UV2100 spectrophotometer at 25 °C, according to the P_i assay of Fiske and SubbaRow (1925). Throughout the text V is the turnover rate, moles of ATP hydrolyzed per mole of S-1 × seconds or minutes. In all cases the rates were measured at least three times and were plotted after subtracting the rates of S-1 alone.

Analyses of Amino Acid Composition and Sequence. Peptides were hydrolyzed at 110 °C for 20 h in evacuated tubes with distilled 6 N HCl and then analyzed with a JEOL-300 A amino acid analyzer. Automated sequence analyses were performed by using an Applied Biosystems 477A protein–peptide sequencing system. A1′ was sequenced as follows: SDS-solubilized papain S-1(A1′) was subjected to 15% polyacrylamide gel electrophoresis (PAGE) and then electroblotted onto a poly(vinylidene difluoride) membrane (Yuen et al., 1990). The protein bands were stained with Comassie Brilliant Blue; the region of A1′ was excised and sequenced with an ABI-477 A protein–peptide sequencing system.

Isolation of the A1 Peptide Containing the 13 Amino-Terminal Residues (N-pep). A1 was dialyzed overnight against 0.2 M ammonium acetate and 2 mM $MgCl_2$ and then digested by 1/666 (w/w) papain for 15 min at 25 °C. The reaction was quenched by adding 1 mM iodoacetic acid. The solution was separated using reversed-phase HPLC in 0.1%

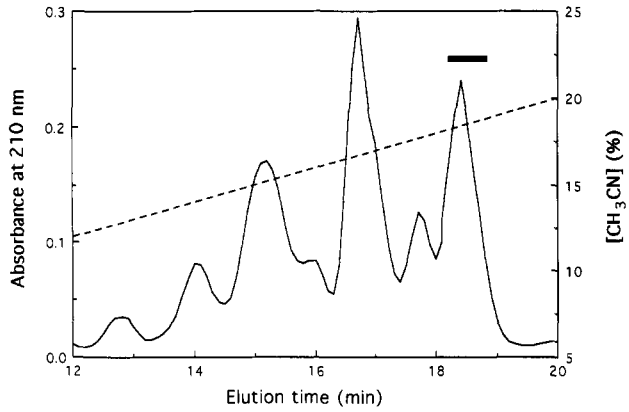

FIGURE 4: HPLC elution profile of papain-digested A1. Papain-digested A1 was applied to a Gasukuro Kogyo Unisil Q C8 packed column (7.6 × 250 mm) and eluted in 0.1% TFA with a linear gradient of acetonitrile at 37 °C, and the absorbance was measured at 210 nm. The flow rate was 2.0 mL/min. The peak indicated by the solid bar was subjected to analyses of amino acid composition, amino acid sequence, ^{1}H-NMR spectroscopy, and LDI-TOF MS.

trifluoroacetic acid (TFA) with a linear gradient of acetonitrile. The concentration of N-pep was determined with a JEOL-300 A amino acid analyzer (see Analyses of Amino Acid Composition and Sequence).

^{1}H-NMR Spectroscopy. The peptide was freeze-dried twice from ^2H_2O and then dissolved to the appropriate concentration in 10 mM imidazole hydrochloride, pH 7.0, containing 5 mM KCl and 2 mM $MgCl_2$ in ^2H_2O. Actin was extracted from the acetone-dried powder of chicken pectoralis muscle by the method of Spudich and Watt (1971) in ^2H_2O.

^{1}H-NMR experiments were performed on a JNM-GX 400 NMR at 400 MHz. Chemical shifts are given in parts per million (ppm), using 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) as the standard.

LDI-TOF Mass Spectrometry. The LDI-TOF mass spectrum was measured with a KOMPACT MALDI III operated at 20 kV, courtesy of Shimadzu Co. (Kyoto, Japan).

Cross-Linking of N-pep and Actin. N-pep (0–50 μM) in 5 mM KCl, 2 mM $MgCl_2$, and 10 mM imidazole hydrochloride, pH 7.0, was mixed with 5 μM actin. Then 0.01 M EDC was added to the mixture. After the mixture was incubated for 2 h at 25 °C, the cross-linking reaction was quenched by addition of excess 2-mercaptoethanol. SDS-solubilized cross-linked products were electrophoresed on a low-bis PAGE composed of 17% acrylamide and 0.0765% bis(acrylamide) (Kagawa et al., 1988) and were stained with Comassie Brilliant Blue.

Peptide Synthesis. A peptide consistent with the 10 amino-terminal residues of the A1 of chicken skeletal muscle (syn-pep) was synthesized except for an amino-terminal alanine substituting N^{α}-trimethylalanine (Me_3Ala), using an ABI-431 A peptide synthesizer. The first 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid was anchored to *p*-(hydroxymethyl)phenoxymethylpolystyrene (HMP) resin. The peptide synthesis and cleavage from the resin were done in an Fmoc cycle and TFA cleavage, respectively, according to the ABI manual. The crude peptide was washed with diethyl ether, extracted with 2 N acetic acid, and lyophilized. Purification of the peptide was carried out using a reversed-phase HPLC column (YMC AM-303 S-5 120 A ODS) in 0.1% TFA with a linear gradient of acetonitrile. The amino acid composition, concentration, and sequence of the peptide were confirmed with a JEOL-300 A analyzer and an ABI-477 A protein–peptide sequencing system.

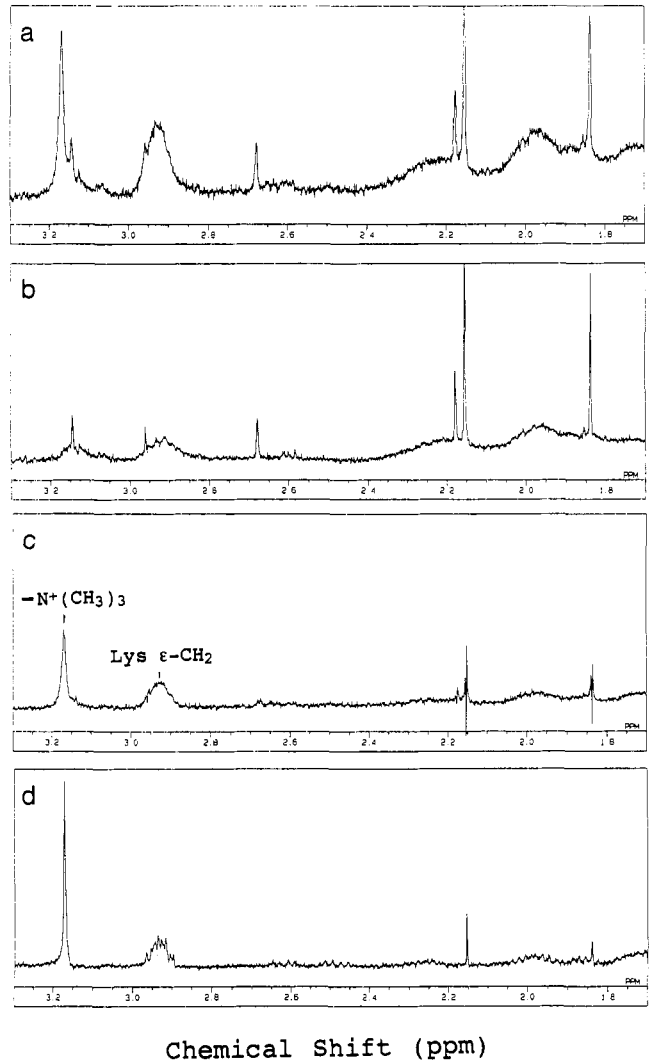

FIGURE 5: ^{1}H-NMR patterns in the interaction of N-pep with F-actin. Samples were dissolved in 10 mM imidazole hydrochloride, pH 7.0 (uncorrected), containing 5 mM KCl and 2 mM $MgCl_2$ in ^2H_2O. Panels: (a) 38 μM N-pep with 37 μM F-actin; (b) 37 μM F-actin alone; (c) difference spectrum obtained by subtracting (b) from (a); (d) 38 μM N-pep alone. The peak widths at half-maximum of the Me_3Ala-$N^+(CH_3)_3$ signals in (c) and (d) were 0.014 and 0.005 ppm, respectively, indicating that the presence of actin broadened the width significantly.

Cross-Linking of Syn-pep and Actin. Syn-pep (0–200 μM) was cross-linked with 5 μM actin as described in Cross-Linking of N-pep and Actin. EDC-treated actin and the EDC-cross-linked syn-pep–actin product were isolated using a gel-filtration HPLC (Figure 7a,b) and were analyzed using both an amino acid sequencer and a low-bis PAGE composed of 17% acrylamide and 0.0765% bis(acrylamide).

RESULTS

Actin-Activated ATPase Activities of Papain S-1 Isoenzymes. The primary structures of A1 and A2 are identical over 141 carboxyl-terminal residues, but A1 possesses an additional 41 residues at its amino-terminal region. Although the additional amino-terminal residues of A1 had been thought to be responsible for the functional difference between S-1(A1) and S-1(A2), the most important region influencing the difference remains to be elucidated. To find the region, we obtained three types of papain S-1 isoenzymes, recombined papain S-1(A1) composed of heavy chain and A1 (Figure 1c,

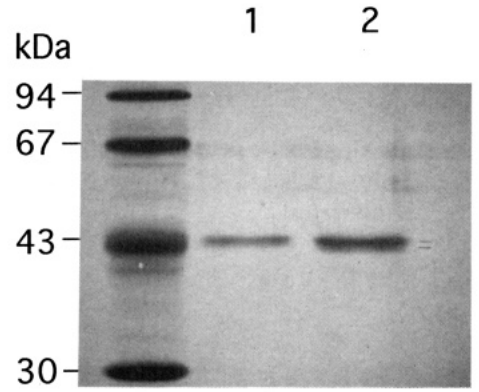

FIGURE 6: SDS–PAGE profile indicating the binding of N-pep with actin. Actin (5 μM) was treated with 10 mM EDC in the absence (lane 1) and presence of 50 μM N-pep (lane 2). SDS-solubilized cross-linked products were electrophoresed on low-bis concentration acrylamide gel containing 17% acrylamide with 0.0765% bis-(acrylamide). The first lane shows the molecular mass standards.

lane b-1), papain S-1(A1′) composed of heavy chain and A1′ (Figure 1c, lane a-1), and papain S-1(A2) composed of heavy chain and A2 (Figure 1c, a-2), and then compared their actin-activated ATPase activities. The $[\text{activity}]^{-1}$ vs $[\text{actin}]^{-1}$ plots for papain S-1(A1′) were similar to those for papain S-1(A2) but different from those for papain S-1(A1), which had a lower K_m for actin and lower V_{max} (Figure 2). Yamamoto and Sekine (1980) reported that A1 of rabbit skeletal muscle myosin was cleaved into smaller pieces by papain somewhere among the additional amino-terminal residues; a papain cleavage site at Lys-17 was suggested (Cardinaud, 1982). In A1 of chicken skeletal muscle myosin, we determined the amino-terminal amino acid sequence of chicken A1′ (Materials and Methods) and compared it with the complete amino acid sequence of chicken A1 to find the papain cleavage site. The sequence analysis of chicken A1′ gave Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro-Ala-Lys. We therefore concluded that chicken A1 was papain-cleaved, at least, between Ala-13 and Ala-14 (Figure 3). The known cleavage-site specificity of papain thus could not predict the present cleavage site. From these results, the region consisting of the 13 amino-terminal residues of A1 seems to be especially important for the kinetic difference between S-1(A1) and S-1(A2).

Table 1: Amino Acid Composition of the N-Terminal Peptide (N-pep) Isolated as in Figure 4

amino acid	residues/molecule[a]
Asp	1.01 (1)
Ala	3.86 (4)
Val	0.93 (1)
Lys	3.84 (4)
Pro	2.36 (2)
total	12

[a] The numbers in parentheses were determined from the 13 N-terminal amino acid sequence of A1 (see Figure 3).

Isolation of the A1 Peptide Containing the 13 Amino-Terminal Residues (N-pep). We tried to isolate N-pep to confirm the papain cleavage site and to investigate the role of the amino-terminal region of A1 in the acto–S-1 interaction. To obtain N-pep, we digested A1 by papain and separated the digests using a reversed-phase HPLC (Figure 4). The amino acid composition of the fraction indicated by the solid bar in Figure 4 was compatible with the 13 amino-terminal amino acid sequence of A1 (Table 1 and Figure 3). The ^{1}H-NMR spectrum of the fraction gave a single peak at 3.2 ppm assignable to the *N*-methyl protons of N^{α}-trimethylalanine [Me_3Ala-$N^+(CH_3)_3$] (Figure 5d). Although we sequenced the fraction with an amino acid sequencer, no phenylthiohydantoin (PTH) amino acid peak was detected (data not shown), apparently because of the modification of the amino terminus of the peptide. The LDI-TOF mass spectrum of the fraction, $M_r = 1336$, corresponded to the mass of the 13 amino-terminal residues of A1 (data not shown). From these results, we concluded that the solid-bar fraction was the 13 amino-terminal residue peptide of A1 (N-pep). The isolation of N-pep confirmed the papain cleavage site of A1 described as above.

Studies on N-pep–Actin Interaction. To investigate the interaction between N-pep and actin, we examined the ^{1}H-NMR spectrum of N-pep in the presence and absence of filamentous actin (F-actin). The ^{1}H-NMR spectrum of N-pep alone is shown in Figure 5d. Prominent in the spectrum are resonances correlated with Lys-ϵ-CH_2 ($\delta = 2.9$ ppm) and Me_3-Ala-$N^+(CH_3)_3$ ($\delta = 3.2$ ppm) as expected from the amino acid composition (Table 1). Panels a and b of Figure 5 show the ^{1}H-NMR spectra of N-pep in the presence of F-actin and of F-actin alone, respectively. Figure 5c is the subtraction [(a) – (b)], i.e., which shows the ^{1}H-NMR spectrum of N-pep

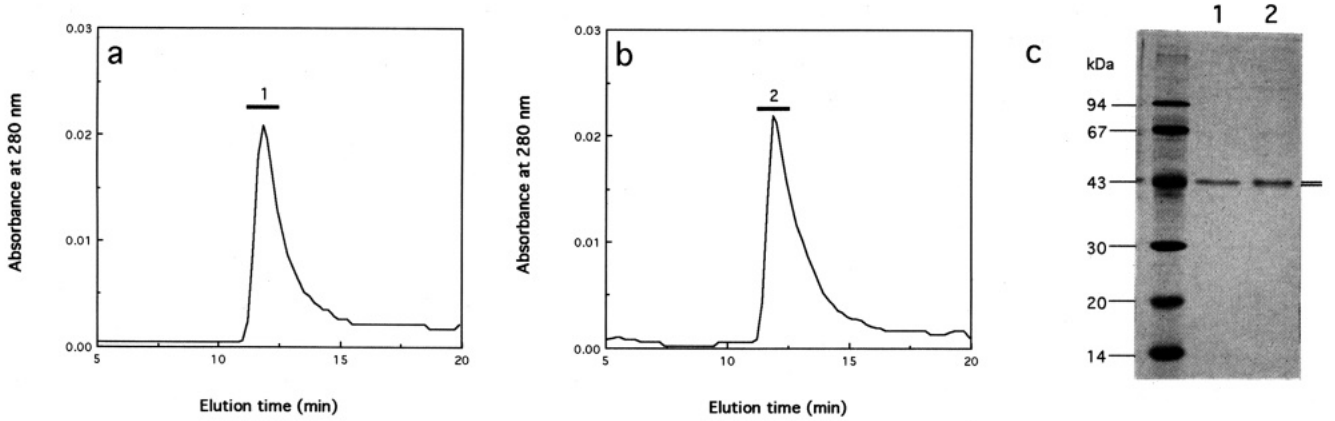

FIGURE 7: (a and b) Gel-filtration HPLC elution profile of EDC-treated actin in the absence (a) and presence of 200 μM syn-pep (b). Actin (5 μM) was treated with 10 mM EDC in the absence and presence of 200 μM syn-pep. EDC-treated samples were applied to a Tosoh TSK-GEL 3000 SW column (30.0 × 0.75 cm) and eluted in 0.1 M ammonium acetate at room temperature, and the absorbance was measured at 280 nm. The flow rate was 0.5 mL/min. The fractions indicated by solid bars as 1 and 2 were examined with SDS–low-bis PAGE as shown in panel c. (c) Low-bis concentration polyacrylamide gel electrophoresis on 17% acrylamide with 0.0765% bis(acrylamide) in the presence of SDS of the pooled fractions shown in panels a and b. The first lane shows the molecular mass standards. Lanes: 1, fraction 1 in panel a; 2, fraction 2 in panel b.

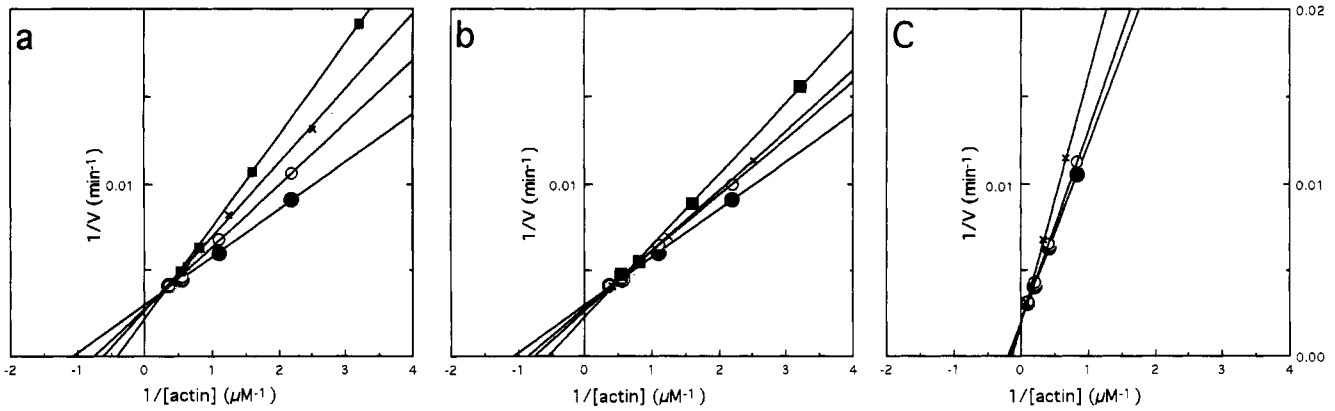

FIGURE 8: (a) Effect of N-pep on the actin-activated ATPase activity of chymotryptic S-1(A1). The [activity]$^{-1}$ vs [actin]$^{-1}$ plots of the activities in the absence (●) and presence of N-pep at concentrations of 3 (○), 6 (×), and 12 μM (■). For 0 μM N-pep, K_m = 0.9 μM and V_{max} = 5.6 s^{-1}. For 3 μM N-pep, K_m = 1.3 μM and V_{max} = 6.2 s^{-1}. For 6 μM N-pep, K_m = 1.6 μM and V_{max} = 6.6 s^{-1}. For 12 μM N-pep, K_m = 2.6 μM and V_{max} = 8.0 s^{-1}. (b) Effect of syn-pep on the actin-activated ATPase activity of chymotryptic S-1(A1). The [activity]$^{-1}$ vs [actin]$^{-1}$ plots of the activities in the absence (●) and presence of syn-pep at concentrations of 3 (○), 6 (×), and 12 μM (■). For 0 μM syn-pep, K_m = 0.9 μM and V_{max} = 5.6 s^{-1}. For 3 μM syn-pep, K_m = 1.2 μM and V_{max} = 6.0 s^{-1}. For 6 μM syn-pep, K_m = 1.3 μM and V_{max} = 6.2 s^{-1}. For 12 μM syn-pep, K_m = 1.8 μM and V_{max} = 7.2 s^{-1}. (c) Effect of N-pep on the actin-activated ATPase activity of chymotryptic S-1(A2). The [activity]$^{-1}$ vs [actin]$^{-1}$ plots of the activities in the absence (●) and presence of N-pep at concentrations of 6 (○) and 12 μM (×). For 0 μM N-pep, K_m = 5.3 μM and V_{max} = 8.7 s^{-1}. For 6 μM N-pep, K_m = 5.8 μM and V_{max} = 8.7 s^{-1}. For 12 μM N-pep, K_m = 7.7 μM and V_{max} = 8.9 s^{-1}. Actin-activated ATPase activity for S-1 isoenzymes was measured at 25 °C using 0.1 μM S-1 isoenzymes with variable actin in 5 mM KCl, 2 mM $MgCl_2$, and 10 mM imidazole hydrochloride, pH 7.0.

in the presence of F-actin. It is noteworthy that the Me_3-Ala-$N^+(CH_3)_3$ signal is broadened by a factor of 3 in the presence of F-actin (Figure 5c) in comparison with N-pep alone (Figure 5d), indicating that an interaction of N-pep with F-actin may occur.

The interaction of N-pep with actin was further examined by cross-linking (Figure 6). Actin (5 μM) was treated by EDC in the absence and presence of 50 μM N-pep as described in Materials and Methods. In the absence of N-pep there was only a band of actin in the SDS–low-bis PAGE (Figure 6, lane 1), while in cross-linking with 50 μM N-pep another band appeared additionally (Figure 6, lane 2), suggesting the cross-linking of N-pep to actin.

In the next, we cross-linked syn-pep (a peptide fragment corresponding to the 10 amino-terminal residues of A1 of chicken skeletal muscle without the trimethylation of the α-amino group of alanine; see Materials and Methods) with actin. The gel-filtration HPLC profiles of EDC-treated 5 μM actin in the absence and presence of 200 μM syn-pep were not distinguishable (Figure 7a,b). However, there was a difference between fraction 1 and fraction 2 in panels a and b of Figure 7 on the low-bis PAGE (Figure 7c). Fraction 1 was composed of a single component (Figure 7c, lane 1), while the fraction 2 was composed of two components (Figure 7c, lane 2) as was observed in the use of N-pep (Figure 6, lane 2). Six cycles of amino acid sequence analysis of fraction 2 gave Ala-Pro-Lys-Lys-Asp-Val, being compatible with the amino-terminal sequence of syn-pep. We did not determine the yield of the cross-linked product, since the separation of the two components was not complete. The sequence analysis of fraction 1 detected no PTH-amino acid residue, apparently because of acetylation of the amino terminus of actin. EDC treatment of syn-pep alone did not give any detectable band on the SDS–PAGE. These results suggested that the product in fraction 2 consisted of both syn-pep and actin. This may indicate that syn-pep could be cross-linked with actin, supporting the binding of N-pep to actin showin in Figure 6.

Binding of N-pep to actin was also supported by a cosedimentation study. A significant amount of N-pep cosedimented with F-actin (data not shown). Since the equilibrium between bound and free forms of N-pep to actin may change during sedimentation, we determined the apparent K_i of N-pep for actin-activated ATPase activity in the following experiment.

Effect of N-pep on the Actin-Activated ATPase Activity of S-1. To clarify the role of the N-pep region of A1 and the significance of the trimethylation of the α-amino group of A1 in the acto–S-1(A1) interaction, we measured the actin-activated ATPase activities among the chymotryptic S-1 isoenzymes in the absence and presence of N-pep or syn-pep. The ATPase activities of the S-1 isoenzymes alone were unaffected by the addition of N-pep or syn-pep (data not shown). In the presence of actin, both the K_m and the V_{max} of the activities for S-1(A1) increased, approximating those for S-1(A2) as the concentrations of either N-pep or syn-pep were increased (Figure 8a,b). Assuming a simple competitive inhibition, we deduced the apparent K_i of N-pep and syn-pep for actin-activated ATPase activity from the results. The deduced apparent K_i of N-pep (6.8 μM actin) was about half that of syn-pep (13.5 μM actin) (Figure 9), suggesting that the affinity of N-pep for actin is significantly higher than that of syn-pep, probably due to the trimethylation of the α-amino group. Contrasting with S-1(A1), the effect of the addition of N-pep on the actin-activated S-1(A2) ATPase activities was very small (Figure 8c), indicating that N-pep selectively inhibits the interaction between A1 and actin.

DISCUSSION

The additional 41 residues of A1 contain a basic amino-terminal region (residues 1–8) followed by a repetitious region of alanyl-prolyl residues (residues 14–27). From the results of ^{1}H-NMR spectroscopy, we found that the 13 amino-terminal amino acid residues of A1 can alone interact with actin without the repetitous Ala-Pro region (Figure 5). This conclusion is supported by experiment of EDC cross-linking of actin with N-pep (Figure 6). Moreover, we observed that the V_{max} and the K_m for papain S-1(A1′) containing the Ala-Pro region were almost identical to those for papain S-1(A2) and remarkably different from recombined papain S-1(A1) including the basic intact amino-terminal region (Figure 2). These results indicate that the kinetic differences between S-1(A1) and S-1(A2) in V_{max} and K_m reported previously for

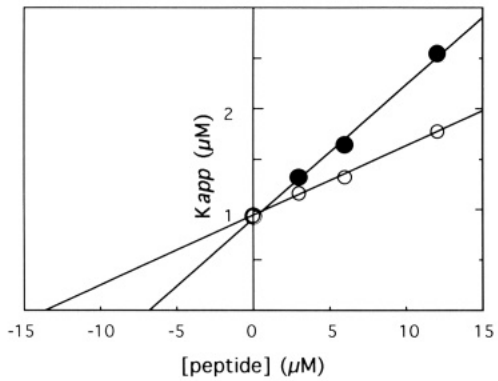

FIGURE 9: Secondary plots of the apparent K_m for the actin-activated ATPase as obtained in Figure 8a (●) and Figure 8b (○) as a function of the concentrations of the peptides. If a simple competitive inhibition is assumed, the relation between the apparent K_m for S-1 (K_{app}) and the apparent K_i for peptide to actin is given by the equation $K_{app} = K_m[1 + [P]/(\text{apparent } K_i)]$, where [P] represents the concentration of peptide. Hence, the intercept on the abscissa gives the apparent K_i for peptide to actin. For N-pep, apparent K_i = 6.8 μM. For syn-pep, apparent K_i = 13.5 μM.

actin-activated ATPase activities (Weeds & Taylor, 1975) are mainly due to the presence of the basic amino-terminal piece but not to the repetitious Ala-Pro residues or other amino acid residues. Although the role of the Ala-Pro region has been undefined by our work, Abillon et al. (1990) postulated from conformational calculations that the repetitious Ala-Pro region may extend the basic amino-terminal region from S-1 to actin with an antenna-like flexibility.

From the apparent K_i values, we showed that N-pep inhibits the binding of S-1(A1) to actin more strongly than syn-pep in actin activation of ATPase activity (Figures 8a,b and 9). N-pep and syn-pep are identical except that N-pep has a positively charged N^{α}-trimethylalanine at the amino terminus and three additional nonpolar residues of alanine at the carboxyl terminus. Because it has been implied that the interaction between actin and the amino-terminal region of A1 is electrostatic [by the results of carbodiimide cross-linking studies (Sutoh, 1982; Yamamoto & Sekine, 1983)], the difference in K_i between N-pep and syn-pep could seem to result not from the additional three residues of alanine but from the N^{α}-trimethylalanine present. These results suggest that trimethylation of the α-amino group of the amino-terminal alanine together with other basic lysyl residues enhances the interaction between actin and A1. Although the physiological meaning of the enhancement shall be examined in a more physiological system, i.e., in muscle fiber, this is the first report that the 13 residues region of A1 can directly interact with actin.

Figure 10 is a diagram of the actin–S-1 interaction. The present results indicate that the small amino-terminal cluster of basic amino acid residues (A site) binds the a site on actin molecules. The binding of A to a may strengthen the interaction between actin (b site) and S-1 (B site) in the presence of ATP, thus lowering the K_m for the actin-activated ATPase and its maximum turnover rate. The isolated A (N-pep) may occupy the a site and inhibit A in S-1(A1) from interacting with a in the actin, in the presence of ATP (Figure 8). Recently Lowey et al. (1993) demonstrated that myosin with A1 moves actin filaments relatively slower than myosin with A2. The A–a interaction was apparently expressed in the actin–myosin mobility assay. The sliding speed between actin and myosin filaments may be modified through the A–a interaction. That is, the A–a interaction may have a suppressive effect on the intermediate transition of the actin–

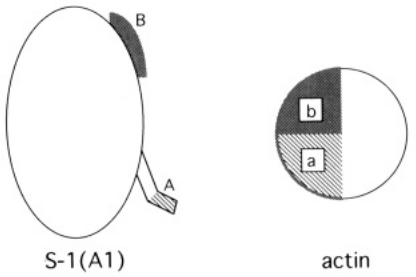

FIGURE 10: Diagram of actin–S-1 binding sites. Site b in the actin molecule binds site B in myosin heavy chain. Site a is to be occupied by the amino-terminal cluster of basic amino acid residues in alkali 1 light chain (site A), depending on the ionic strength of the medium. Binding of A to a strengthens the binding of B to b, resulting in a high binding affinity for acto–S-1 and a slower turnover rate of actin-activated ATPase activity.

myosin–nucleotide tertiary complex. This diagram does not conflict with the previous reports showing that myosin alkali light chains are not essential for the ATP hydrolysis of S-1 ATPase (Wagner & Giniger, 1981; Sivaramakrishnan & Burke, 1982).

Three-dimensional structures of both actin and S-1 have been shown (Kabsch et al., 1990; Holmes et al., 1990; Milligan et al., 1990; Rayment et al., 1993a,b). The residues Tyr^{626}–Gln^{647} on myosin can interact with amino acid residues located between Asp^{1} and Glu^{4} and including Asp^{24} and Asp^{25} on actin. DasGupta and Reisler (1989) showed that site-specific antibodies directed against the first seven amino-terminal residues on actin inhibited the binding of S-1 to actin, resulting in the inhibition of actin-activated ATPase of S-1. Sutoh et al. (1991) showed that mutation of Asp^{1} and Asp^{4} of *Dictyostelium* actin to His residues, as well as Glu^{99} and Glu^{100} to His residues (Johara et al., 1993), made actin filaments exhibit ATP-dependent myosin binding but be unable to suppport movement in an *in vitro* motility assay. Residues Lys^{567}–His^{578} on myosin are also shown to interact with residues Tyr^{91}–Gln^{100} on actin (Rayment et al., 1993b). Bonafé et al. (1993) showed a 50-kDa segment of S-1 cross-linked to actin via Arg^{95} with *p*-azidophenylglyoxal. The interactions of the S-1 heavy chain with actin demonstrated as above can be described as the B–b interaction in Figure 10.

Sutoh (1982) showed that A1 could be cross-linked to the carboxyl-terminal region of actin. Trayer et al. (1987) showed that the amino terminus of A1 was found close (<1.5 nm) to the spin-labeled Cys^{374} on actin by NMR studies. The conformation of the amino terminus of A1 in S-1 was not yet elucidated in three dimensions (Rayment et al., 1993a). These previous reports imply that the a site may be located in the vicinity of the carboxy terminus of actin. Where the amino-terminal 13 residue peptide of A1 binds the actin molecule needs to be determined more in detail and to be characterized in a more organized system, i.e., muscle fibers, in the near future.

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