Properties of the Alkali Light-Chain–20-Kilodalton Fragment Complex from Skeletal Myosin Heads†

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ABSTRACT: We have developed a rapid and reproducible procedure widely applicable to the preparation of pure aqueous solutions of the complex between an alkali light chain and the COOH-terminal heavy-chain fragments of skeletal myosin chymotryptic subfragment 1 (S-1) split by various proteases. It was founded on the remarkable ethanol solubility of these complexes. A systematic study of the ethanol fractionation of the tryptic (27K–50K–20K)-S-1 (A2) showed the NH_2-terminal 27K fragment to behave like a specific protein entity being quantitatively precipitated at a relatively low ethanol concentration. Only the 20K peptide–A2 complex remained in solution when the S-1 derivative was treated with exactly 4 volumes of ethanol in the presence of 6 M guanidinium chloride. At a lower ethanol concentration, a soluble mixture of 50K and 20K peptides together with the light chain was obtained. The isolated 20K fragment–A2 system containing a 1:1 molar ratio of each component was investigated by biochemical and ^1H nuclear magnetic resonance (NMR) techniques to highlight its structure and the interaction of the 20K heavy-chain segment with F-actin and with the light chain. During the treatment of the complex with α-chymotrypsin, only the 20K peptide was fragmented in contrast to its stability within the whole S-1. The binding of F-actin to the complex led, however, to a strong inhibition of its chymotryptic degradation. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide cross-linking of F-actin to the complex produced covalent actin–20K peptide only, the amount of which was lower relative to that observed with the entire split S-1. The binding constant of actin to the complex determined by sedimentation at various strengths was 100-fold weaker than for native S-1 and was similar to that reported previously for the 20K peptide preparation without light chain. The ^1H NMR experiments indicated that the interaction of the 20K peptide–A2 complex with F-actin led to changes in the tertiary structure of the A2 light chain consequent on actin binding to the 20K peptide. We conclude that an actin-induced conformational change could be transmitted through the 20K segment to the homologous 150-residue COOH-terminal region of the alkali light chains. This feature may have implications in the mechanism of energy transduction by the actomyosin complex.

The pioneering investigations on the limited tryptic digestion of rabbit skeletal myosin subfragment 1 (S-1)[1] (Balint et al., 1978; Mornet et al., 1979; Yamamoto & Sekine, 1979) have established that the myosin head heavy chain is constituted by the three fragments denoted "27K", "50K", and "20K". These were encountered in the S–1's from various myosins (Flink & Morkin, 1982; Marianne-Pêpin et al., 1983; Szentkiralyi, 1984). They were produced by several different proteases and might correspond to domainlike structures within the native S-1 moiety (Mornet et al., 1981a, 1984; Chaussepied et al., 1983; Applegate & Reisler, 1983).

The first peptide to be sequenced was the COOH-terminal 20K fragment containing the pair of reactive SH_1–SH_2 thiols (Elzinga & Collins, 1977; Gallagher & Elzinga, 1980). The chemical cross-linking of actin to this 20K unit in the skeletal S-1 and to the homologous 25K fragment of smooth myosin S-1 (Mornet et al., 1981a,b; Marianne-Pêpin et al., 1985) and the conformational changes induced by nucleotides in this region (Wells & Yount, 1982) suggest that it may play an important role in the coupling between the actin and ATPase sites during energy transduction (Botts et al., 1984). Moreover, it is likely that the 20K heavy-chain element is interacting with the alkali light chains. This was suggested by intramolecular cross-linking experiments on skeletal S-1 (Labbé et al., 1981), electron microscopy of a proteolytic fragment from scallop myosin (Winkelman et al., 1984), and binding studies between the light subunits and the isolated tryptically cut heavy chain of rabbit myosin S-1 (Burke & Kamalakannan, 1985).

To further understand the multiple functionalities of this particular region of the myosin head, we recently attempted to isolate a soluble 20K peptide–alkali light-chain complex

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[1] Abbreviations: S-1, myosin chymotryptic subfragment 1; acto–S-1, actomyosin–S-1; A1, alkali light-chain 1; A2, alkali light-chain 2; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; ^1H NMR, proton nuclear magnetic resonance spectroscopy; CPMG, Carr–Purcell–Meiboom–Gill; DTT, dithiothreitol; DTE, dithioerythritol; TSS, sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4; AEDANS, *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; 20K fragment, 20-kilodalton fragment; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; NaDodSO$_4$, sodium dodecyl sulfate; pPDM, *N,N'-p*-phenylenedimaleimide; EDTA, ethylenediaminetetraacetic acid.

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from trypsin-split skeletal myosin S-1. Our efforts were based on the original observation of Balint et al. (1975) that the tryptic 20K fragment of skeletal S-1 (referred to as 21K peptide) exhibits an α-helical character from its properties of resolubilization after precipitation with ethanol. Using a combination of guanidinium chloride dissociation and ethanol fractionation similar to that employed previously by Perrie and Perry (1970) on intact myosin, we could reproducibly obtain a 20K peptide–light-chain preparation in which the 20K component was still able to interact with F-actin. The preliminary description of these investigations (Kassab et al., 1981; Kassab, 1982) encouraged Morales and collaborators to isolate the 20K peptide alone without light chains (Muhlrad & Morales, 1984).

Because the isolated 20K–light-chain system is obviously more resembling S-1 than the 20K devoid of light subunits, we describe here in detail its preparation and some of its biochemical properties. We also report for the first time on the conformation of the isolated 20K peptide and its interaction properties with the A2 light chain and with F-actin, as analyzed by ^1H nuclear magnetic resonance spectroscopy. This technique was successfully applied previously to native S-1 and acto–S-1 to probe the internal motility of the myosin head and its modulation by actin (Highsmith et al., 1979; Prince et al., 1981).

Materials and Methods

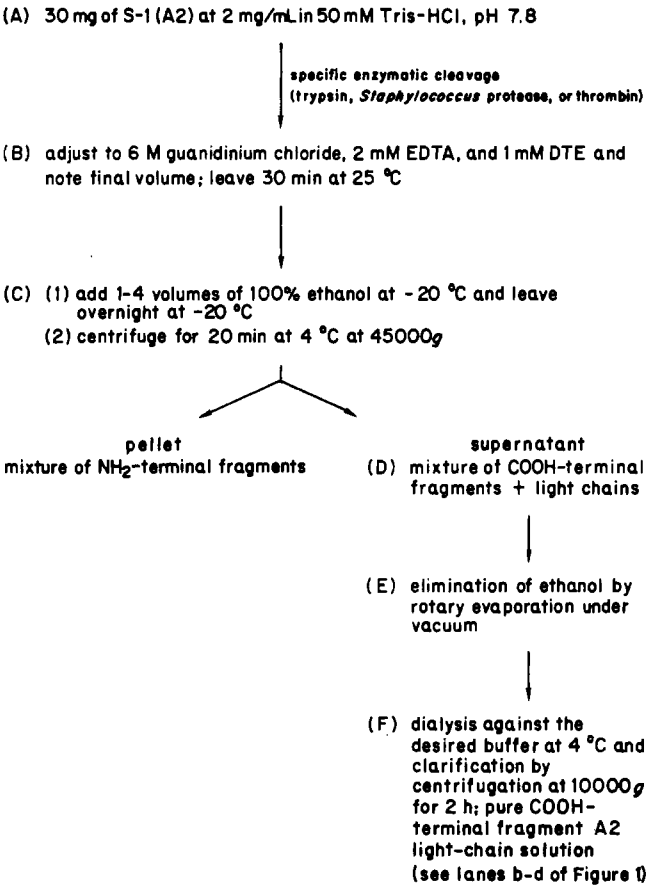
Chemicals. Ultrapure guanidinium chloride was supplied by Mann Research. Deuterium oxide (99.8%) was from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Urea-d_4 was obtained from Aldrich. α-Chymotrypsin and trypsin [treated with L-(tosylamido)-2-phenylethyl chloromethyl ketone] were purchased from Worthington Biochemical Corp. All other chemicals were of the highest analytical grade.

Protein Preparations. Subfragment 1 (A1) and subfragment 1 (A2) from rabbit skeletal muscle myosin were isolated as described by Weeds and Taylor (1975). F-actin was prepared as in Eisenberg and Kielly (1974).

A2 light chain was isolated as previously described (Henry et al., 1985). Split (27K–50K–20K)-S-1 was obtained by tryptic digestion of chymotryptic S-1 as described previously (Mornet et al., 1981a). The preparation of the COOH-terminal 20K fragment–light-chain complex was carried out as indicated in Scheme I. In the last step (F), the solution was supplemented with sucrose (0.2 M) and β-mercaptoethanol (5 mM) and then centrifuged at 100000g for 2 h at 4 °C. The clear solution was stored at 4 °C. Most experiments were performed on freshly prepared samples (1–3-days old). The concentration of 20K peptide in the preparation was estimated by amino acid analysis of the content in 3-methylhistidine assuming 1 residue/mol of 20K peptide (Gallagher & Elzinga, 1980) and using a Beckman Model 119 B analyzer with the elution program reported by Mornet et al. (1980). Proteins were prepared for NMR as follows: F-actin was dialyzed against 10 mM phosphate buffer, 0.02% NaN_3, and 0.25 mM DTT in ^2H_2O (pH 7.4). The 20K peptide–A2 solutions were dialyzed overnight against 1 L of 10 mM phosphate, pH 7.4, and 1 mM DTT to remove the sucrose, concentrated by Aquacide if necessary, and then dialyzed against 3 × 100 mL of 10 mM phosphate, pH 7.4, 0.5 mM DTT, 0.02% NaN_3, and 0.1 mM TSS in ^2H_2O.

Proteolytic Digestions. The solution of 20K peptide–light chain was submitted to digestion with α-chymotrypsin and trypsin in 0.1 M Tris-HCl buffer, pH 7.5 at 25 °C, in the absence and presence of a 2-fold excess of F-actin. A weight ratio of protease to 20K peptide of 1:200 was employed. The time courses of the proteolytic reactions were followed by gel electrophoresis.

Scheme I: General Procedure for Isolation of the C-Terminal Heavy-Chain Fragment–Light-Chain Complex from Fragmented Skeletal S-1



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

Before use, dialysis bags were boiled at 100 °C for 5 min.

Binding Experiments. The affinity of actin for the 20K peptide–light-chain preparation was measured at different KCl concentrations by sedimentation in a Beckman airfuge essentially as described by Chalovich and Eisenberg (1982) and Chaussepied et al. (1986b). Native S-1 was used as control. S-1 (5 μM) or 20K fragment (3 μM) was mixed with varying concentrations of F-actin (0–7 μM) in 40 mM Tris-HCl buffer, pH 7.6, 5–110 mM KCl, 2.5 mM $MgCl_2$, and 0.5 mM DTE. After sedimentation at 178000g for 35 min at 22 °C, the fraction of S-1 remaining in the supernatant was determined from its K^+-ATPase activity and by densitometric measurements of the 95K heavy-chain band present on $NaDodSO_4$ electrophoretic gels. For the 20K peptide, the latter procedure was employed with estimation of the 20K band relative to a 20K–light-chain standard.

NMR Methods. ^1H NMR spectra were obtained at 300 MHz on a Brucker spectrometer using quadrature detection and at a sample temperature of 25 °C. A short (0.25 s) preacquisition pulse was used to suppress the residual water resonance, having first established that presaturation at the power levels used did not affect the signal intensity of the protein resonances. The two-pulse Hahn spin-echo and the Carr–Purcell–Meiboom–Gill (CPMG) spin-echo techniques used have been previously described (Jardetzky & Roberts,

Table I: Percent of S-1 Heavy-Chain Peptides and A2 Light Chain Present in the Supernatant after Ethanol Fractionation[a]

ethanol (volumes)	peptide			
	50K	27K	20K	A2
0	100	100	100	100
1.5	60	0	40	30
3.0	40	0	60	40
4.0	0	0	50	50

[a] The values were obtained by densitometric scanning of the gels presented in Figure 1.

1981). An interpulse delay of 5 ms was typically used in the latter experiments in order to filter resonance intensity on the basis of relaxation time, the spectrum of signals with notably short relaxation times being obtained by difference spectroscopy (see legend to Figure 5).

Titrations were carried out by incremental additions of stock solutions. The pH values quoted are direct meter readings, uncorrected for isotope effects. Chemical shifts are in ppm from internal sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSS). Quoted protein concentrations for the 20K fragment–A2 light-chain complex are based on the 3-methylhistidine content of the 20K fragment (see above).

Results

Isolation of the COOH-Terminal 20K Heavy-Chain Fragment–Alkali Light-Chain Mixture from Trypsin-Split S-1. When the skeletal chymotryptic S-1 (A2) was fragmented with trypsin into (27K–50K–20K)-S-1 (A2) and then submitted to the guanidinium chloride treatment outlined in Scheme I, a soluble solution is reproducibly obtained in the last step (F) containing a mixture of the 20K heavy-chain peptide and the intact A2 subunit. If the S-1 (A1) isoenzyme was the starting material, the approach yielded the 20K peptide together with the tryptic breakdown products of the A1 light chain with masses in the range 23K–17K (Hayashi, 1972; Cardinaud, 1979; Mornet et al., 1979). The procedure presented in Scheme I is of general use as it could be applied also to the preparation of various COOH-terminal heavy-chain fragments deriving from the limited digestion of skeletal S-1 by other proteases. Thus, we have also obtained soluble and actin-interacting solutions of the 22K fragment produced by cleavage of native S-1 with *Staphylococcus* protease (Chaussepied et al., 1983; Applegate & Reisler, 1983; Mornet et al., 1984) and of the 30K peptide generated by the thrombic digestion of 5-thio-2-nitrobenzoate–S-1 (Chaussepied et al., 1986a).

The degree of purity of the COOH-terminal heavy-chain fragment–light-chain preparation reached in step F was highly dependent on the extent of elimination of the two other NH_2-terminal heavy-chain fragments as contaminants during the step of ethanol fractionation (step C). Because this part of the procedure is critical, we have investigated qualitatively and quantitatively the influence of ethanol concentration on the aqueous solubility of each of the three heavy-chain fragments of trypsin-cut (27K–50K–20K)-S-1 (A2). This was done by analyzing on electrophoretic gels the peptide composition of the different solutions produced in the final step F by the addition of 1–4 volumes of ethanol in step C (Figure 1); concomitantly, we measured the relative amount of each peptide species present in these solutions (Table I). The results show that the NH_2-terminal 27K fragment exhibits a striking behavior as compared to the other heavy-chain components. It was readily and totaly precipitated by the addition of only 1–1.5 volumes of ethanol (Figure 1, lane b, and Table I); in contrast, approximately half of the 50K fragment remained in solution at up to 3 volumes of added ethanol (Figure 1, lane c, and Table I). Only the addition of exactly 4 volumes of ethanol could lead to the complete precipitation of the large central fragment and to the production of a soluble 20K fragment–light-chain A2 mixture essentially depleted of NH_2-terminal peptides (Figure 1, lane d, and Table I). Denistometric scanning of the electrophoretograms corresponding to several 20K peptide–A2 preparations showed that these two stable components were present in an almost 1:1 molar ratio. Efforts to separate the two peptides under nondissociating conditions, using further ethanol fractionation, gel filtration, and DEAE-cellulose chromatography, were unsuccessful. On the other hand, cross-linking experiments, to be reported elsewhere, indicated the formation of a covalent product between the 20K fragment and the A1 light chain (Labbé et al., 1985); also, the cosedimentation of F-actin with the 20K peptide–A2 or the 20K peptide–A1 preparations showed the presence of both the heavy-chain and light-chain peptides in the pellets (data not shown). The existence in solution of an equilibrium complex between the two isolated components was further suggested by the ^{1}H NMR data reported below.

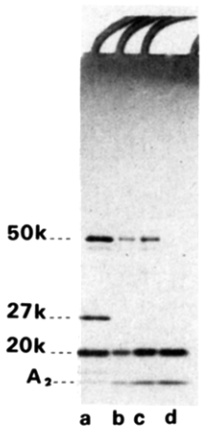


FIGURE 1: Electrophoretic analysis of the peptide composition of different supernatants formed during ethanol fractionation of (27K–50K–20K)-S-1 (A2). As described in Scheme I, the 6 M guanidinium chloride solution of split (27K–50K–20K)-S-1 (A2) was submitted in step C to a precipitation procedure using an increasing amount of ethanol. The renatured protein solutions obtained after dialysis in step F were submitted to gel electrophoresis. Lanes a, b, c, and d correspond to 0, 1.5, 3, and 4 volumes of ethanol employed, respectively.

Interaction of F-Actin with the 20K Peptide–Light-Chain Mixture. The actin binding ability of the 20K fragment would be dependent on the extent of its refolding in the presence of the light chain during the dialysis step F. We therefore tested the renaturation efficiency of this step by analyzing the susceptibility of a 20K–light-chain (A1 + A2) preparation to chymotrypsin (Figure 2). This protease does not act at all on the alkali light chains or on the heavy chain in native S-1. Figure 2A shows that the A2 subunit together with the 23K tryptic product of A1 was still refractory to this protease and thus has recovered, most probably, a nearly native conformation. In contrast, the 20K fragment was rapidly cut into small peptides with mobility lower than the 17K–A2 light chain; the fragment was also cleaved similarly by trypsin. Previously, the isolated, light-chain-free 20K peptide was also found sensitive to proteolysis (Muhlrad & Morales, 1984). Such a lability of the heavy-chain peptide both in the presence and in the absence of the light chains could be expected because of the fact that the 20K segment structure was likely to be stabilized by intramolecular contacts with the other heavy-chain regions in the native S-1. Interestingly, however, Figure 2B shows that the addition of F-actin to the 20K

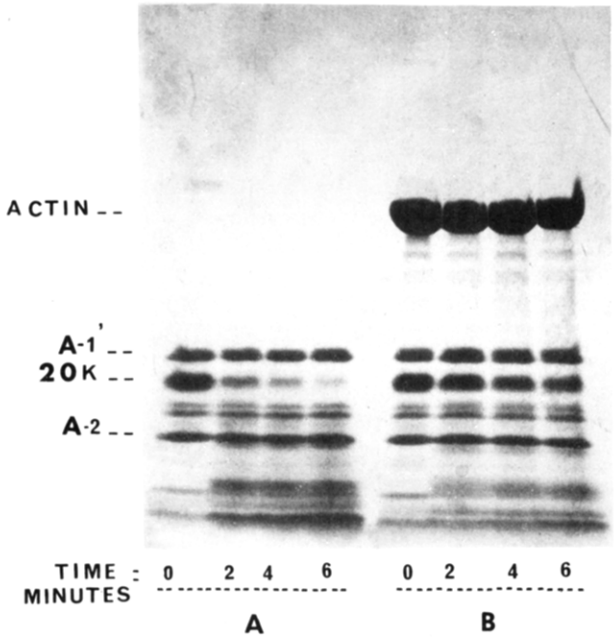


FIGURE 2: Influence of F-actin on chymotryptic digestion of the isolated 20K fragment–light-chain complex. The 20K fragment–light-chain solution (2 mg in 20K peptide/mL) was incubated with chymotrypsin in the absence (A) and in the presence (B) of F-actin (2-fold excess over 20K peptide); other conditions were as described under Materials and Methods. At the times indicated, samples were analyzed by electrophoresis on 5–18% polyacrylamide slab gels.

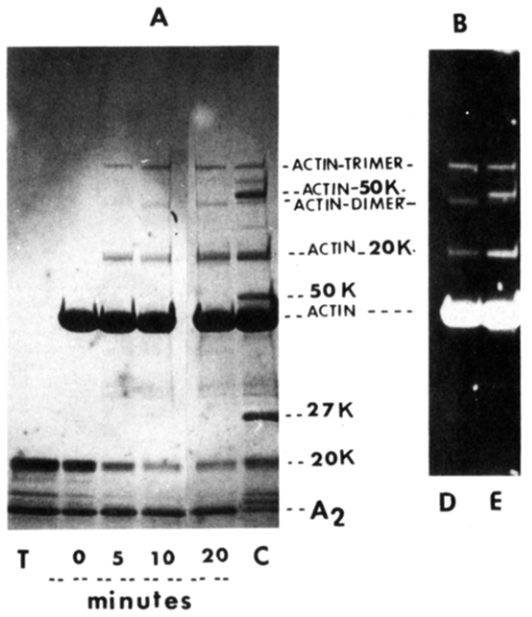


FIGURE 3: Cross-linking of F-actin to the 20K fragment–A2 light-chain complex. (A) Time course of the cross-linking reaction between F-actin and 20K peptide–A2 (2 mg of 20K peptide/mL) in 50 mM 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.5) in the presence of 10 mM EDC; F-actin was added at a molar ratio to 20K peptide of 2. At the times indicated, samples were submitted to $NaDodSO_4$ gel electrophoresis; proteins bands were stained with Coomassie blue. T = 20K peptide–A2 light-chain preparation; C = 20 min. EDC cross-linking between F-actin and trypsin-split (27K–50K–20K)-S-1 under similar conditions. (B) The gels in (A) were viewed under UV light using fluorescent F-actin labeled with 1,5-IAEDANS. (D) and (E) represent the fluorescent patterns of 20-min cross-linking reactions for actin–20K peptide–A2 and actin–(27K–50K–20K)-S-1, respectively.

peptide–light-chain preparation, in a molar ratio of actin to 20K fragment of 2, induced a dramatic change in the digestibility of the 20K entity by chymotrypsin. The rate of its degradation was strongly decreased and a significant amount of the peptide remained intact in spite of the occurrence of several chymotrypsin-sensitive bonds along the 20K fragment sequence (Gallagher & Elzinga, 1980). The protective effect of actin can be understood by the formation of a tight complex between actin and the 20K fragment with alteration of the accessibility or environment of a number of apolar side chains in the peptide. Alternatively, the folding of the 20K fragment might be improved by its interaction with actin.

Finally, the interaction of F-actin with the 20K peptide–A2 preparation was compared to the association between F-actin and the whole (27K–50K–20K)-S-1 (A2) using carbodiimide-catalyzed cross-linking (Figure 3). The 20K peptide solution yielded the expected covalent actin–20K peptide band clearly identified on the electrophoretic gels by the incorporation of the fluorescence of AEDANS–F-actin (Figure 3A,B). Like the split S-1 control, it did not give rise to any A2–actin cross-linked product. However, under essentially identical experimental conditions, the 20K preparation produced a smaller amount of the cross-linked actin–20K peptide species as compared to the nondissociated (27K–50K–20K)-S-1 (A2). This could result from the lower affinity of actin for the isolated peptide. Indeed, the data of the binding experiments shown in Table II indicated that at μ = 24–130 mM, the binding constant of actin to a 20K–(A1 + A2) solution was about 100-fold weaker as compared to that determined for S-1 (A1 + A2) whereas the actin affinity for trypsin-cut S-1 (A1 + A2) is only 10-fold weaker (Fukarama & Arata, 1984). The binding data we obtained for the 20K peptide–light-chain mixture are in agreement with those reported for the light-chain-free 20K peptide (Muhlrad & Morales, 1984). They indicate that the affinity of the isolated 20K element for actin is not much influenced by the presence or absence of the alkali light chain.

Table II: Comparative Binding Constants (M^{-1}) for Complexes between Actin and Native S-1 and between Actin and the 20K Fragment–Light-Chain Preparation at Different Ionic Strengths[a]

actin complexes	ionic strength (mM)		
	24	52	130
20K (A″1 + A2)	4.4×10^5	3.9×10^4	8×10^3
S-1 (A1 + A2)	4.5×10^7	7.6×10^6	1.2×10^6

[a] The values of the binding constants were obtained from the slopes of Scatchard representations. Binding experiments were performed as described under Materials and Methods. A″1 denotes the tryptic degradation product of A1.

^{1}H NMR Study of the 20K Peptide–A2 Complex. The structural and dynamic aspects of the interaction between actin and the 20K–A2 complex may be monitored by ^{1}H NMR spectroscopy. Initial studies were undertaken in order to determine the structural integrity of the 20K–A2 complex in light of ^{1}H NMR spectral data for the intact S-1 (A2) myosin isoenzyme (Prince et al., 1981). The ^{1}H NMR spectrum of the native complex is shown in Figure 4. Spectral information about the individual proteins in this binary complex is available once signals which derive from each component are separately identified. This can first be achieved by comparison with the spectrum of the isolated A2 light chain (Figure 4A). Several aromatic and $-CH_3$ group signals are resolved in the spectrum of the isolated light chain with chemical shift positions shifted away from the major resonances. These altered resonance energies are dictated by the different electronic environments of the corresponding groups imposed by the configuration of the A2 light chain that prevails in solution. Only certain of these shifted signals can be identified in homologous positions in the spectrum of the 20K–A2 complex (Figure 4A).[2] Two

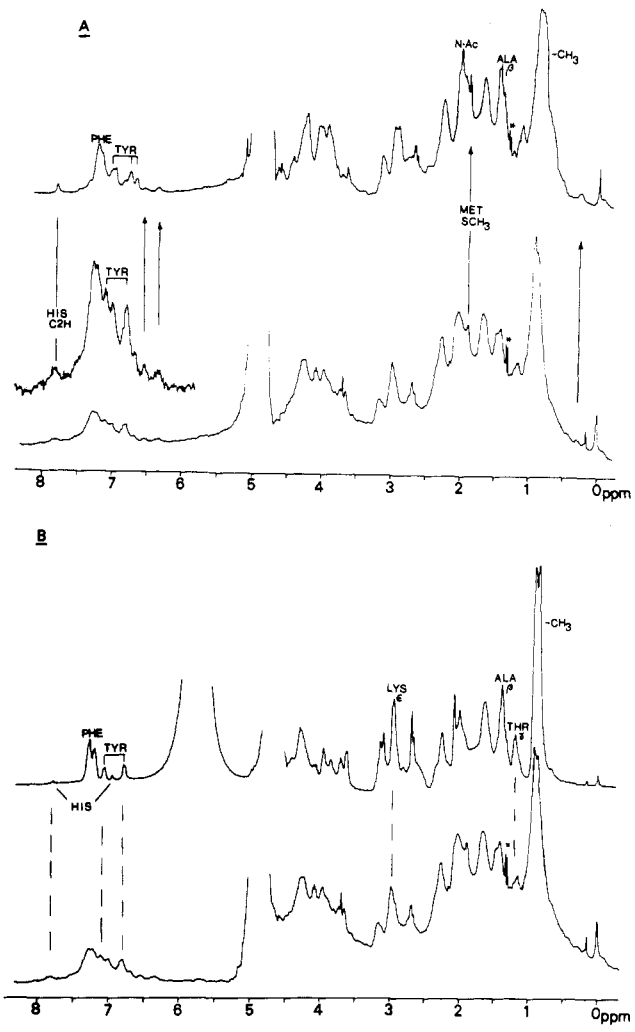


FIGURE 4: Spectral comparison of native and denatured 20K fragment–A2 light-chain complex with isolated A2 light chain. (A) ^{1}H NMR spectra of the 20K–A2 complex, pH 7.5, 80 μM (lower trace), and the isolated A2 light chain, 320 μM, at the same solution pH (upper trace). Signals of the A2 light chain with homologous spectral positions are identified. (B) ^{1}H NMR spectra of the 20K–A2 complex (80 μM) in the absence (lower trace) and presence of 8 M urea-d_4 (upper trace), pH 7.5. Signals in the denatured protein can be assigned to residue type on the basis of chemical shift position. The proteins were equilibrated to 10 mM phosphate buffer, pH 7.4, (uncorrected), containing 0.02% NaN_3, 0.1 mM TSS, and 0.5 mM DTT in ^2H_2O, and spectra were collected at 25 °C.

subsets of spectral window thereby emerge. Those signals of A2 that are directly recognized in the 20K–A2 spectrum presumably derive from the region(s) of the light chain that are relatively unperturbed by interaction between the two proteins, while those signals whose spectral shift is altered by complex formation reflect changes in the configuration of the alkali light chain. Further inspection of the remaining signals similarly identifies specific resonances corresponding to each of the two subsets, e.g., a methionine $-SCH_3$ signal and the *N*-acetyl resonance of A2 [1.90 and 2.06 ppm, respectively (Figure 4A), and resolved by two-pulse spin-echo methods (not shown)] appear in both isolated A2 and 20K–A2 spectra, while the signals of the side chains of His-109 and/or His-145 of the light chain (Frank & Weeds, 1974) are notably broadened in the spectrum of 20K–A2. The light-chain configuration must therefore adjust upon complex formation. Attachment between the proteins, however, leaves specific segments of the light chain with mobility independent of the overall (tumbling) motion of the complex.

Previous ^{1}H NMR spectral studies (Prince et al., 1981) have shown that the heavy-chain portion of the myosin subfragment 1 isoenzyme, S-1 (A2), adopts a relatively inflexible configuration whose ^{1}H NMR spectral resonances are generally broad. To determine the extent to which signals of the 20K moiety contribute to the spectrum of the 20K–A2 complex, the protein complex was denatured by titration with urea to a final concentration of 8 M. The latter spectrum (Figure 4B) shows that a marked increase in signal intensity occurs during the titration concomitant with the loss of the broad resonance envelope underlying the spectrum of the 20K–A2 complex (Figure 4B). The well-defined resonances in the presence of urea show relative intensities in keeping with the residue composition of the 20K–A2 complex, and by comparison with denaturation experiments on the isolated light chain, it is readily inferred that a substantial proportion of the signals of the 20K moiety are notably broad. This observation agrees well with the spectral data for the intact S-1 (A2) isoenzyme and indicates that the molecular framework of the 20K component possesses a high degree of structural organization with few segments whose mobility is comparable to that of the light chain.

^{1}H NMR Study of the Interaction of F-Actin with the 20K Peptide–A2 Complex. Resonance intensity deriving from the 20K component was identified by using a combination of spin-echo methods and difference spectroscopy to filter out the broader signals in the spectrum of the 20K–A2 complex on the basis of their short relaxation times (T_2). Such a set of difference spectra during titration with actin are shown in Figure 5A to illustrate the effect of interaction with actin on the spectral envelope for the 20K component. Specific regions of this envelope are perturbed by the binding of actin: e.g., ~3 ppm, typical of lysine ϵ-CH_2; ~2.1–2 ppm, typical of Met S–CH_3. The envelope intensity, however, and hence the overall mobility of many side chains of the 20K fragment, is by no means quenched upon complex formation with actin. These alterations in spectral features are also observed in the direct spectra obtained during the titration (Figure 5B).

Interestingly, it is noteworthy that during titration of the 20K–A2 complex with actin, signals deriving from aromatic groups of the A2 light chain were also perturbed. The influence of actin on these signals is shown Figure 6A together with the spectra obtained upon titration of the isolated light chain with actin (Figure 6B). No interaction occurs between actin and the isolated light chain as previously observed in other studies (Henry et al., 1985). These ^{1}H NMR observations therefore indicate that the interaction between actin and the 20K component is transmitted to the alkali light chain.

Discussion

The production and isolation of a protein complex comprising the light chains associated to a heavy-chain fragment of the myosin head are not without precedent. Recently, such

[2] Secondary shifts away from those characteristic of a particular chemical group arise from the through-space influence of electronic shielding effects induced by neighboring groups indicative of a relatively well-defined environment. It is assumed here that spectral homology in resonance position of specific signals in the spectrum of the isolated A2 molecule and in the spectrum of the 20K–A2 complex is a reflection of structural integrity for the corresponding regions of the A2 molecule. The possibility exists that the secondary shift correspondence in the two spectra is accidental and that spectral homology occurs for different residues in each spectrum. The possibility of such accidental correspondence cannot be discounted at this stage of the investigation. If this was the case, however, then association of the light chain with the 20K moiety results in a more marked modification of the configuration of the light chain than is directly apparent.

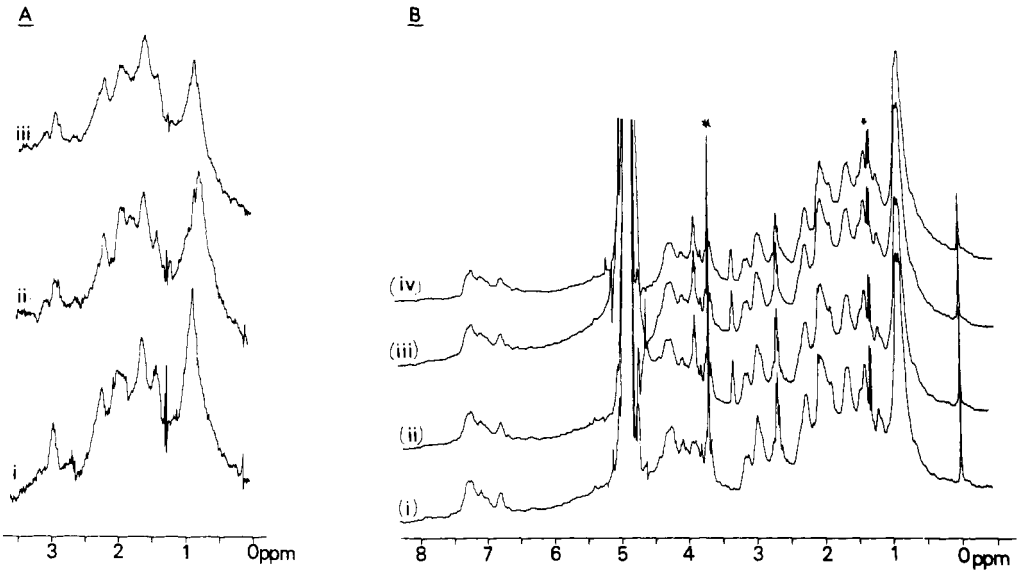


FIGURE 5: Effect of actin addition on the ^1H NMR spectra of the 20K fragment–A2 light-chain complex. (A) ^1H NMR aliphatic spectral envelope corresponding to the relatively broad signals deriving from the 20K component during titration of the complex with actin. These spectra were obtained by subtraction of data obtained with the CPMG spin-echo technique (τ = 5 ms) from the normal spectra [see (B)]. Similar envelopes were obtained upon different spectroscopy using CPMG spectra accumulated by using 1- and 5-ms delays. Without actin (i) and with molar ratios of complex to actin of 4.5:1 (ii) and 1:1 (iii). Concentration of the 20K–A2 complex was 70 μM. (B) Conventional spectra of the 20K–A2 complex (70 μM) obtained during titration with actin. Without actin (i) and with molar ratios of complex to actin of 4.5:1 (ii), 2.2:1 (iii), and 1:1 (iv); signals at 1.31 and 3.70 ppm (indicated with asterisks) were unknown impurities probably originating from dialysis tubing and from guanidinium chloride, respectively. The signal intensity of broad intensity of F-actin does not contribute significantly to any of these spectra [cf. Prince et al. (1981) and Highsmith et al. (1979)]. The proteins were equilibrated to 10 mM phosphate buffer, pH 7.4 (uncorrected), containing 0.02% NaN_3, 0.1 mM TSS, and either 0.5 mM DTT (20K peptide–A2 complex) or 0.25 mM DTT (F-actin) in ^2H_2O, and spectra were collected at 25 °C.

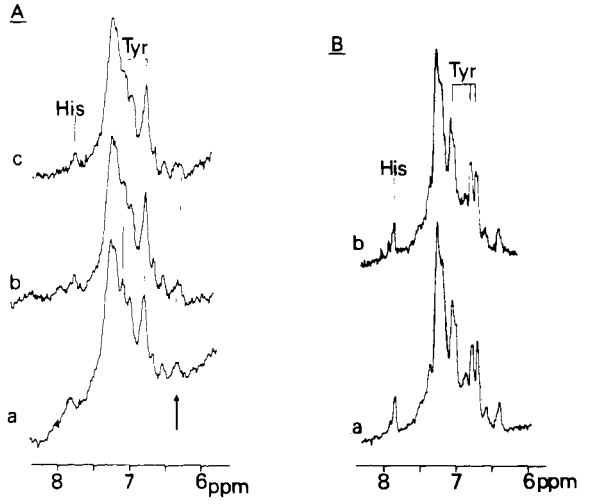


FIGURE 6: Comparison of the effect of actin on the A2–light-chain-derived ^1H NMR signals in the 20K fragment–A2 light-chain complex and isolated light chain. (A) Conventional spectra of the aromatic region of the 20K–A2 complex (70 μM) upon addition of actin showing spectral shifts of the resonances deriving from A2. Without actin (a) and with molar ratios of actin to complex of 0.5 (b) and 1 (c). (B) Aromatic region of the isolated A2 light chain (70 μM) upon addition of actin. (a) Spectrum of A2; (b) spectrum of A2 + F-actin (mole ratio 1:1). The spectrum of F-actin does not contribute significantly to any of these spectra (Prince et al., 1981; Highsmith et al., 1979). The proteins were equilibrated to 10 mM phosphate buffer, pH 7.4 (uncorrected), containing 0.02% NaN_3, 0.1 mM TSS, and either 0.5 mM DTT (20K peptide–A2 complex) or 0.25 mM DTT (F-actin) in ^2H_2O, and spectra were collected at 25 °C.

a complex containing the regulatory and –SH light chains combined to a heavy-chain peptide was purified from scallop myosin S-1 after an extensive tryptic digestion (Szentkiralyi, 1984; Bennet et al., 1984). However, the nature of the heavy-chain component has not been characterized although it was certainly derived from the COOH terminus of the S-1 heavy chain. In contrast, the present complex such as the 20K peptide–A2 preparation represents a well-defined protein system with respect to the S-1 heavy-chain peptide. It is also the first complex to be isolated from a vertebrate myosin S-1. Most importantly, this complex is more equivalent to the intact S-1 as the heavy-chain peptide exhibits association properties not only with the light chain but also with F-actin. Comparative studies are under way on the complex from chicken gizzard myosin.

On the basis of the amount of 20K peptide specifically determined by amino acid analysis, the yield of the complex preparation was approximately 30%, a value similar to that found for the light-chain-free peptide (Muhlrad & Morales, 1984). We applied the experimental approach described in Scheme I not only to native S-1 but also to S-1 chemically modified on the 20K heavy-chain region by the blocking of the reactive SH_1 thiol or of both the SH_1 and SH2 sulfhydryls with bulky groups such as AEDANS and pPDM (*N,N'-p*-phenylenedimaleimide). However, these two thiol modifications have led to a dramatic decrease of the yield of soluble 20K peptide to less than 5%. Thus, the stability of the isolated heavy-chain fragment appears to depend critically on the integrity of the S-1 reactive thiols. It might be possible that selective changes in the ethanol fractionation step could improve the isolation of the modified 20K peptide–light-chain complex. In addition to the 20K peptide, the large central 50K fragment showed also a relatively good ethanol stability, in particular when less than 4 volumes of ethanol were employed. The production of soluble 50K material in the renaturation step (Figure 1) is consistent with the recent observations of Muhlrad et al. (1985) describing the partial refolding of denaturant-free 50K peptide. In contrast, the NH_2-terminal 27K fragment behaved quite differently. First, 95% of the peptide could be precipitated at only 1 volume of ethanol; second, attempts made to achieve its resolubilization and renaturation like the 20K peptide were so far unsuccessful. Similar results

were obtained after its conversion into the shorter 22K derivative which forms upon tryptic cleavage of the parent peptide at Arg-23 in the presence of Mg^{2+}-ATP (Mornet et al., 1985). These findings suggest a particular conformation for the 27K segment the folding of which seems much less autonomous than that presented by the two other heavy-chain fragments.

During the final renaturation step, the 20K peptide structure has regained a sufficient degree of refolding which allows the expression of the two known functionalities of this region in S-1, namely, the binding to the light chains and to F-actin. We employed a combination of ^{1}H NMR spectroscopy and biochemical approaches to have greater insight into these two important properties. It was reported recently that the association of the alkali light chain to the S-1 heavy chain required the structural integrity of a 2K peptide at the COOH terminus of the 20K segment (Burke & Kamalakannan, 1985). Thus, it may be reasonably anticipated that the light-chain recognition site within this area of the isolated 20K peptide has recovered a proper fold. The interaction of the A2 light chain with the 20K peptide is accompanied by specific changes in the A2-associated resonances in the ^{1}H NMR spectrum of the 20K peptide–A2 complex. It suggests that the light-chain structure undergoes conformational changes induced by complex formation with the heavy-chain peptide. So far, only the structure of the light chains in the isolated state has been investigated (Alexis & Gratzer, 1979). The present ^{1}H NMR study provides the first indication that parts of this structure could be changed upon binding of the light subunit to the S-1 heavy chain.

Recent findings based on light-chain exchange indicate that, in the absence of the LC2 light chain, there is a dynamic binding equilibrium between the alkali light chain and the myosin or S-1 heavy chains (Pastra-Landis & Lowey, 1985). We observed that the Phe and Tyr spectrum typical of A2 was markedly decreased in the 20K peptide–A2 ^{1}H NMR spectrum. If we assume that the 6.70 ppm peak in the complex is a ring-shifted Tyr of A2 and the 6.81 ppm peak is due to the sum of the four Tyr residues of the 20K peptide and the other two Tyr residues of A2, then one gets a 6.81:6.70 ppm ratio from 20K peptide–A2 spectra, of about 12:1 intensity ratio, indicating that 30 ± 6% of A2 is free, not bound to the 20K fragment; knowing the protein concentration used (70 μM), we calculated a binding constant $K_b = 1.2 \times 10^5$ M^{-1}. Thus, A2 and 20K peptide would seem to be in binding equilibrium in solution. The influence of the 20K peptide on the Tyr spectrum of A2 is probably relevant to the observation that the chemical modification of two tyrosyl residues in the alkali light chains suppressed their ability to reassociate to the S-1 heavy chain, suggesting the involvement of tyrosyl groups in the heavy-chain–alkali light-chain interaction (Burke & Wang, 1982). Finally, it is noteworthy that the *N*-acetyl signal (2 ppm) was as mobile in A2 as in A2 complexed to the 20K peptide. This suggests that the N-terminal part of A2 must be mobile in the 20K peptide–A2 complex.

The binding of actin to the 20K segment of the native S-1 heavy chain was previously shown to involve contacts with the N-terminal lysine residues of the 20K peptide (Mornet et al., 1981; Sutoh, 1983) and unknown amino acid side chains around the SH_1–SH_2 groups (Katoh et al., 1985). The latter region is thought to contribute principally to the attachment of S-1 to actin in the rigor state (Katoh & Morita, 1984; Katoh et al., 1985). The ^{1}H NMR spectra indeed show that resonances of Lys ϵ-CH_2 in the heavy-chain peptide–A2 spectra were affected by actin.

Furthermore, close inspection of Figure 5 indicates that additional perturbations in the ^{1}H NMR spectra of the 20K peptide–A2 complex occur as it binds actin over those already cited, e.g., at 0.9 ppm, typical of Val, Ile, and Leu CH_3; at 1.4 ppm, typical of Ala β-CH_3. At least some of these appear to originate from the 20K component of 20K–A2 complex (Figure 5A). It should be emphasized that actin has no influence on the ^{1}H NMR spectrum of the isolated A2 light chain. We can conclude, therefore, that the 20K peptide–A2 complex is likely to bind actin at selected points on the surface of the 20K moiety. The observed perturbation of signals deriving from side chains of hydrophobic residues is consistent with proposals (Katoh & Morita, 1984; Katoh et al., 1985) that hydrophobic forces are involved in the strong rigor interaction between actin and the S-1 heavy chain mediated by the 20K segment.

The overall side-chain mobility of the 20K peptide (Figure 5A) was not severely quenched by actin. In marked contrast, interaction between actin and intact S-1 (A2) leads to a substantial loss in signal intensity associated with reduced segmental mobility within an already relatively inflexible structural framework for the heavy chain (Prince et al., 1981). These spectral effects are also observed in studies of actin binding to S-1 following tryptic digestion (Goodearl et al., 1985). In view of the apparently specific interaction between the 20K–A2 complex and actin ($K_b = 5 \times 10^5$ M^{-1}, as shown in Table II), it is plausible that the region of the intact heavy chain whose internal mobility is constrained by actin binding corresponds to the 50K portion which contains the second class of actin binding sites. This would indicate that the 20K and 50K domains of S-1 behave as virtually independent entities. ^{1}H NMR spectral studies on intact S-1 where the SH_1 and SH_2 of the 20K portion have been cross-linked have, however, shown that relatively little reduction in internal mobility occurs upon binding actin (Goodearl et al., 1985). The 20K and 50K domains cannot therefore be considered as independent entities but must rather act cooperatively upon interaction with actin. This would also explain the higher affinity of actin to the 20K fragment within the (27K–50K–20K)-S-1 as illustrated by its greater cross-linking ability relative to that for the isolated fragment.

Finally, the present ^{1}H NMR studies showed for the first time that the actin interaction with the 20K peptide was also communicated to the A2 portion of the complex. To date, the possible role of the common 150-residue COOH-terminal region of the alkali light chains is poorly understood. We interpret the data in keeping with the notion that the common region of the alkali light chains could enable the transmission of coupling energy through distinct regions of the myosin head.

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