THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 26, Issue of June 25, pp. 18271–18277, 1999

Printed in U.S.A.

Size and Charge Requirements for Kinetic Modulation and Actin Binding by Alkali 1-type Myosin Essential Light Chains*

(Received for publication, October 26, 1998, and in revised form, March 9, 1999)

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The alkali 1-type isoforms of myosin essential light chains from vertebrate striated muscles have an additional 40 or so amino acids at their N terminus compared with the alkali 2-type. Consequently two light chain isoenzymes of myosin subfragment-1 can be isolated. Using synthesized peptide mimics of the N-terminal region of alkali 1-type essential light chains, we have found by ^{1}H NMR that the major actin binding region occurred in the N-terminal four residues, APKK. . . . These results were confirmed by mutating this region of the human atrial essential light chain, resulting in altered actin-activated MgATPase kinetics when the recombinant light chains were hybridized into rabbit skeletal subfragment 1. Substitution of either Lys^3 or Lys^4 with Ala resulted in increased K_m and k_{cat} and decreased actin binding (as judged by chemical cross-linking). Replacement of Lys^4 with Asp reduced actin binding and increased K_m and k_{cat} still further. Alteration of Ala^1 to Val did not alter the kinetic parameters of the hybrid subfragment 1 or the essential light chain's ability to bind actin. Furthermore, we found a significant correlation between the apparent K_m for actin and the k_{cat} for MgATP turnover for each mutant hybrid, strengthening our belief that the binding of actin by alkali 1-type essential light chains results directly in modulation of the myosin motor.

Muscle contracts when a molecular motor protein, myosin, hydrolyzes MgATP and uses the energy so released to pull past filaments of its partner protein, actin. Although the mechanism of contraction appears to be broadly similar between muscle types, the way in which it is controlled is not. The universal trigger for contraction is a rise in the intracellular free calcium concentration, but the way in which this is sensed and the way in which the message is transmitted to the active site of myosin varies considerably (see Refs. 1–5). Furthermore, many muscle systems are modulated by additional proteins that fine tune the on/off signaling resulting from calcium ion concentration fluctuations.

Myosin is a hexamer of two 200-kDa heavy chains, two essential light chains (ELC)[1] of 17–21 kDa, and two regulatory light chains (RLC) of 20 kDa. The ELC of vertebrate striated muscle exist in two isoforms: alkali 1 (A1)-like and alkali 2 (A2)-like (6, 7), and the presence of one, or the other, isoform appears to fine tune the contractile properties of the myosin molecule carrying it. This effect has been demonstrated in single muscle fibers (8), in *in vitro* motility assays (9), and in enzyme assays (10), where experiments are generally carried out with the more biochemically amenable myosin subfragment 1 (S1, a soluble, proteolytic fragment of myosin that retains the actin binding and MgATPase activities of the intact molecule). S1 prepared by chymotryptic digestion carries only one ELC and a fragment of the heavy chain (10). Thus, there are two possible S1 isoenzymes, S1(A1) and S1(A2), depending on which ELC isoform is associated with the heavy chain.

Recent mutagenesis studies have established that the extreme N-terminal residues of A1-type ELCs (which differ from A2-like ELCs by having 40-odd additional amino acid residues at the N terminus) encode an actin binding site. We have shown by deletion mutagenesis studies that the site lies within the first 11 residues and that actin binding by these residues results directly in modulation of the kinetic properties of S1 (11). The remaining residues of the N-terminal extension of A1-type ELCs are highly proline-rich and form an extended structure (12) responsible for correctly positioning the actin binding site on the surface of actin (13). In this study we address the role of individual amino acids of the human atrial A1-type ELC (HmAtELC; Ref. 14) in the modulatory event.

Previous NMR studies on the intact rabbit skeletal A1-type ELC have suggested the involvement of one or more Lys residues and the N-terminal trimethylalanine residue in actin binding (15–17). In this report, we show by NMR that the key residues involved in binding to actin are Ala^1, Lys^3, and Lys^4 at the N terminus of the Al-type ELC. This has been confirmed by site-directed mutagenesis where these residues have been substituted and the mutants tested for their ability to cross-link to actin and their effects on K_m and k_{cat} of the actin-activated ATPase of S1 hybrids. Removal of the positive charge at position 3 and 4 gave a kinetic phenotype that was more S1(A2)-like than the wild-type hybrid (which is S1(A1)-like; Ref. 11). These studies show that the k_{cat} is directly controlled by the apparent K_m for actin, further supporting the view that actin binding by A1-type ELCs result directly in kinetic modulation of the myosin motor.

* This work was supported in part by the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ELC, myosin essential light chain; S1, myosin subfragment 1; S1(A1) and S1(A2), myosin subfragment 1 carrying an alkali 1 (A1)- or alkali 2 (A2)-type essential light chain, respectively; HmAtELC, human atrial essential light chain; RLC, myosin regulatory light chain; EDC, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide; Fmoc, 9-fluorenylmethoxycarbonyl; CPMG, Carr-Purcell-Meiboom-Gill; PAGE, polyacrylamide gel electrophoresis; DSS, 2,2-dimethylsilapentane-5-sulfonic acid; TOCSY, total correlation spectroscopy; COSY, correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy.

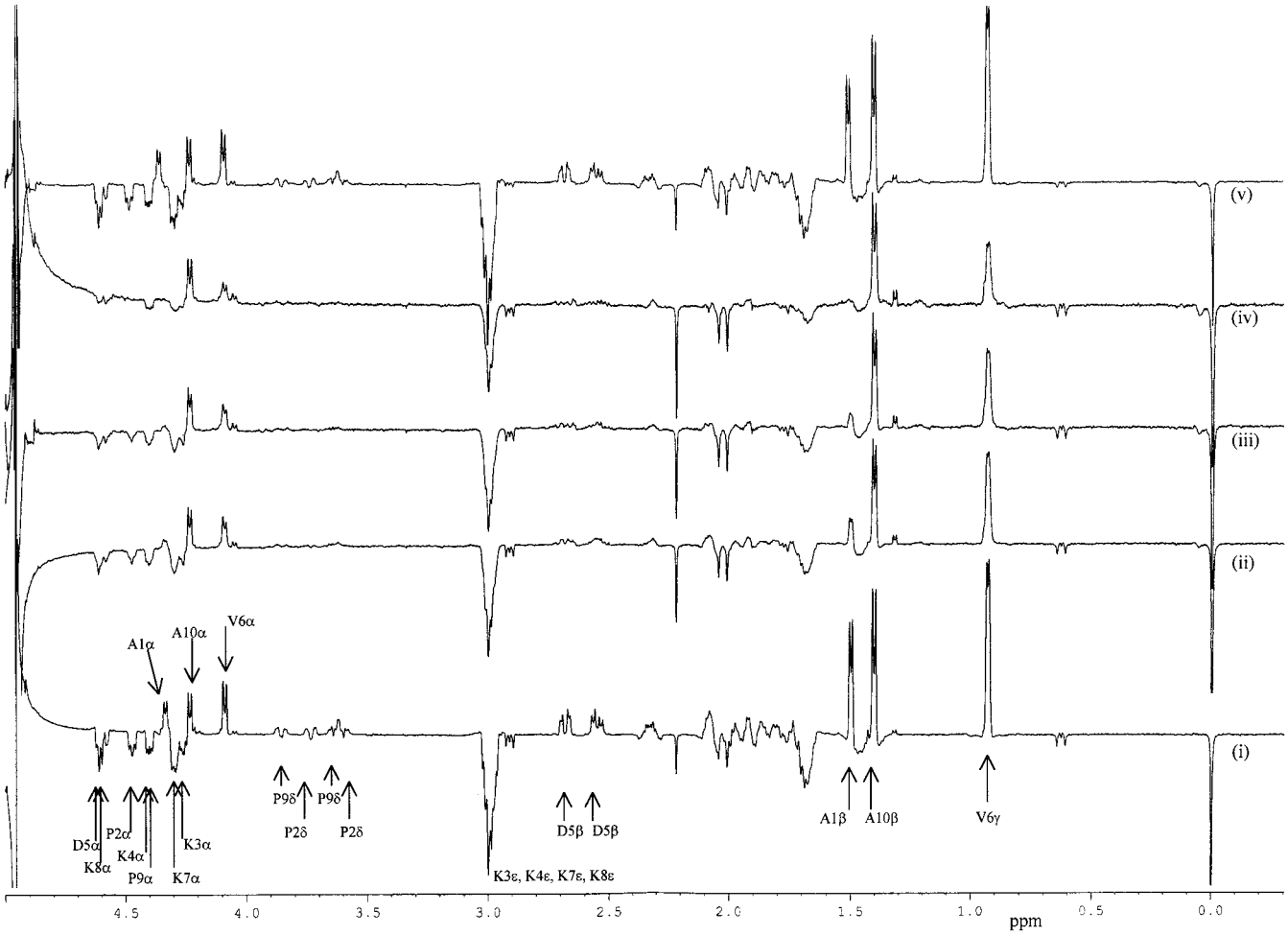

FIG. 1. **Interaction of A1-type ELC peptide 1–10 with F-actin determined by ^{1}H NMR.** CPMG spectra of the N-terminal 10 residues of rabbit skeletal Al-type ELC (NH_3^+-APKKDVKKPA-$CONH_2$) in 5 mM phosphate buffer, pH 7.2, containing 2 mM $MgCl_2$, 0.3 mM NaN_3, 0.1 mM DSS at 285 K. In all experiments the peptide concentration was 500 μM. *i*, peptide alone; *ii*, peptide plus 16.7 μM F-actin (ratio 30:1); *iii*, peptide plus 22.7 μM F-actin (ratio 22:1); *iv*, peptide plus 33.3 μM (ratio 15:1); *v*, *iv* plus 100 mM KCl. In the experiments described in Figs. 1–3, the actin was fully relaxed prior to data collection and is not seen in the spectra.

EXPERIMENTAL PROCEDURES

Production of Expression Vectors Carrying Mutant HmAtELCs—All mutant proteins were expressed from plasmids derived from the T7-based overexpression vector pET-11c (18). The polymerase chain reaction (19) was used to amplify the HmAtELC cDNA (kindly supplied to us by Dr. A. Starzinski-Powitz, Universität Frankfurt Klinikum, Senckenberg Zentrum Pathologie, D-6000, Frankfurt, Germany) in the vector pUC-19, and the various mutations were introduced by using different 5′ oligonucleotide primers that incorporated changes in the appropriate codons. In all cases the primers were designed so as to introduce an *Nde*I restriction enzyme site 5′ to the coding sequence and a *Bam*HI site 3′ to it. Polymerase chain reaction products were digested with these two enzymes and the product of this reaction ligated into *Nde*I/*Bam*HI-cut pET-11c. Plasmids containing these inserts were transformed into *Escherichia coli* BL21(DE3) cells for protein overexpression as described previously for the wild-type HmAtELC (13). Overexpression was monitored by comparing the SDS-PAGE profiles of cell extracts from cells before and after induction with isopropyl-1-thio-β-D-galactopyranoside The sequence of all mutant ELC constructs were verified by double stranded dideoxynucleotide sequencing (20) and the N-terminal sequence checked by amino acid sequencing. The N-terminal amino acid sequences of the first 10 residues of the wild-type and mutant proteins were: HmAtELC, APKKPEPKKE . . . ; HmAtELC(A1V), VPKKPEPKKE . . . ; HmAtELC(K4R), APKRPEPKKE . . . ; HmAtELC(K4A), APKAPEPKKE . . . ; HmAtELC(K4D), APKDPEPKKE . . . ; HmAtELC(K3A), APAKPEPKKE . . .

Protein and Peptide Preparations—Unless otherwise stated, all protein manipulations were carried out at 4 °C. Actin was prepared from muscle acetone powder according to standard techniques (21) and was stored as G-actin freeze-dried from buffer A (5 mM triethanolamine hydrochloride, pH 7.5, 0.2 mM calcium chloride, 0.2 mM ATP, 0.25 mM dithiothreitol) at −20 °C until required. F-actin was prepared from this as described in Ref. 11. Chymotryptic myosin S1 was prepared according to the method of Weeds and Taylor (10) except that SP-Tris Acryl^{GSB} (IBF Biotechnics, France) was used to separate the isoenzymes (S1(A1) and S1(A2)) (22). Aliquots of the purified isoenzymes were shell frozen at −80 °C in the presence of 4 mg of sucrose/mg of S1 and 2 mM dithiothreitol and stored at −70 °C until required.

Recombinant ELCs were purified by ammonium sulfate fractionation and column chromatography on DEAE-Sepharose Fast-Flow (Amersham Pharmacia Biotech) exactly as described previously for the wild-type HmAtELC (11, 13). Purified ELCs were stored as freeze-dried powders at −20 °C until required.

The effect of each mutation on the steady state MgATPase kinetics of S1 was assessed by hybridizing the light chain into rabbit skeletal S1(A2) using the ammonium chloride dissociation method (7) as modified in Ref. 23 and using SP-Tris Acryl to separate the mutant hybrid from the residual S1(A2) (11, 22). Hybrids were concentrated by precipitation in 70% (mass/volume) ammonium sulfate before being taken up in about 1 ml 5 mM triethanolamine hydrochloride, pH 7.5, 0.25 mM dithiothreitol and dialyzed against this buffer overnight. In order that control experiments with rabbit skeletal S1(A1) and S1(A2) would match closely as possible the experiments with hybrid S1, material for controls was obtained by pooling the residual S1(A1) and S1(A2) in hybridization experiments.

The synthesis of peptides representing the N-terminal 10 residues of rabbit skeletal A1-type ELC 1–10, NH_3^+-APKKDVKKPA-$CONH_2$; 1–6, NH_3^+-APKKDV-$CONH_2$; and 5–10, *N*-acetyl-DVKKPA-$CONH_2$, was carried out by Alta Bioscience, University of Birmingham, Birmingham, United Kingdom They were synthesized by standard Fmoc-protected

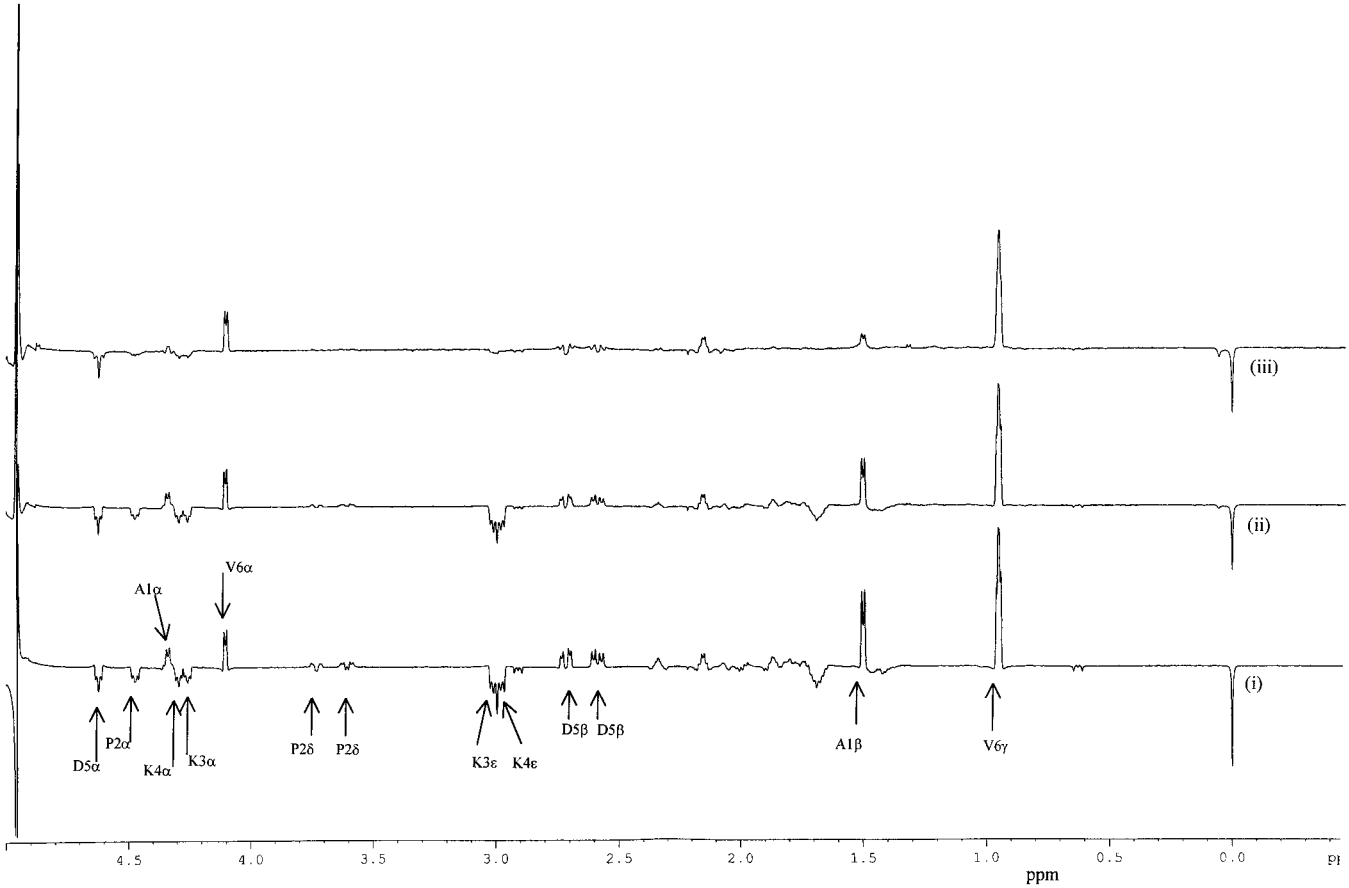

FIG. 2. **Interaction of A1-type ELC peptide 1–6 with F-actin determined by ^{1}H NMR.** CPMG spectra of the N-terminal 6 residues of rabbit skeletal A1-type ELC (NH_3^+-APKKDV-$CONH_2$) in 5 mM phosphate buffer, pH 7.2, containing 2 mM $MgCl_2$, 0.3 mM NaN_3, 0.1 mM DSS at 285 K. Peptide concentration was maintained at 500 μM in all experiments. *i*, peptide alone; *ii*, peptide plus 33.3 μM F-actin (ratio 15:1); *iii*, peptide plus 50 μM F-actin (ratio 10:1).

solid phase methods and were purified by reverse-phase high pressure liquid chromatography on a Vydac C18 column using an acetonitrile gradient in 0.1% trifluoroacetic acid. Their purity was confirmed by protein sequencing and by matrix-assisted laser desorption ionization mass spectrometry, and their concentrations determined by amino acid analysis.

Steady State MgATPase Assays—MgATPase activities were determined by measuring phosphate release (24) in a stopped enzyme assay at 25 °C. Various concentrations of F-actin (in buffer A supplemented with 2.0 mM magnesium chloride; the amount of supplemented buffer A plus F-actin solution was kept constant at 40 μl/ml of assay mix) were mixed with mutant hybrid S1s (concentrations of which varied between 0.04 and 0.17 μM) in 5 mM triethanolamine hydrochloride, pH 7.5, 0.25 mM dithiothreitol. The reaction was initiated by addition of magnesium chloride to 2.5 mM and ATP to 2.0 mM and then processed and analyzed as described in Ref. 11.

Cross-linking—The procedure used for cross-linking ELCs to actin was based on Ref. 25 as detailed in Ref. 11. The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-cross-linked products were then analyzed by SDS-PAGE and identified on the basis of their size and by comparison to previously assigned bands (11, 13). S1 hybrids were cross-linked to actin using a similar protocol except that S1:actin molar ratios were typically 1:4, since increasing the saturation of F-actin with S1 decreases the amount of ELC-actin product (25).

NMR Spectroscopy—All ^{1}H NMR experiments were collected on a Bruker AMX 500 spectrometer. For one-dimensional binding titration experiments, the solution conditions were 500 μM peptide, 5 mM potassium phosphate, 2 mM $MgCl_2$, 0.3 mM NaN_3, 0.1 mM DSS, pH 7.2, at 285 K, with ratios of added F-actin varied between 30:1 and 5:1. Binding was monitored by standard one-dimensional experiments (data not shown) and by Carr-Purcell-Meiboom-Gill (CPMG) experiments with a single spin echo (90°-τ-180°-τ) and a 60-ms refocusing delay (26, 27). A two-dimensional TOCSY experiment was collected for 3.5 mM peptide 1–10 and 325 μM F-actin (ratio 11:1; conditions as above, except KCl was added to 42 mM and the experiments were carried out at pH 6.8). For each peptide, resonances were assigned using a 500 μM sample of peptide and the following two-dimensional experiments; TOCSY (MLEV-17 mixing pulse of 60-ms duration at a spin locking field of 10 kHz) (28), COSY (data not shown), NOESY (data not shown) (500-ms mixing time), ROESY (data not shown) (continuous mixing pulse of 250-ms duration, 3.2-kHz locking field). A very weak presaturation pulse was applied to the water peak during the relaxation delay (1.5 s) and also during the mixing time for NOESY and ROESY experiments.

Analytical Methods—12% SDS-PAGE gels were run at between 35 and 50 mA (constant current) and stained with Coomassie Blue (29). N-terminal protein sequencing was carried out on an Applied Biosystems 473A automated protein sequencer by Alta Bioscience, School of Biochemistry, University of Birmingham. Protein concentrations were estimated from absorbance measurements using the following values: $A_{280,1mg/ml}$(HmAtELC) = 0.22 mg^{-1}·ml; $A_{290,1mg/ml}$(Actin) = 0.63 mg^{-1}·ml and molecular masses: HmAtELC, 21 kDa; actin, 42 kDa. The mutations described in this study were not expected to alter the absorption characteristics or molecular mass of the light chain significantly, and so these values were used for all mutant ELCs.

RESULTS

All striated muscle Al-type ELCs that have been analyzed contain N-terminal Ala (most) or Pro (some cardiac isoforms) that have been post-translationally modified by methylation (15).[2] The recombinant proteins used in this study are not modified in any way (and have been compared with synthesized peptides with a free N terminus). The 9 proton NMR signal arising from the trimethylalanine of rabbit skeletal S1(A1), however, was readily visible in the ^{1}H NMR spectrum of this protein and broadened significantly upon addition of actin (15,

[2] C. A. Fruin and I. P. Trayer, unpublished observations.

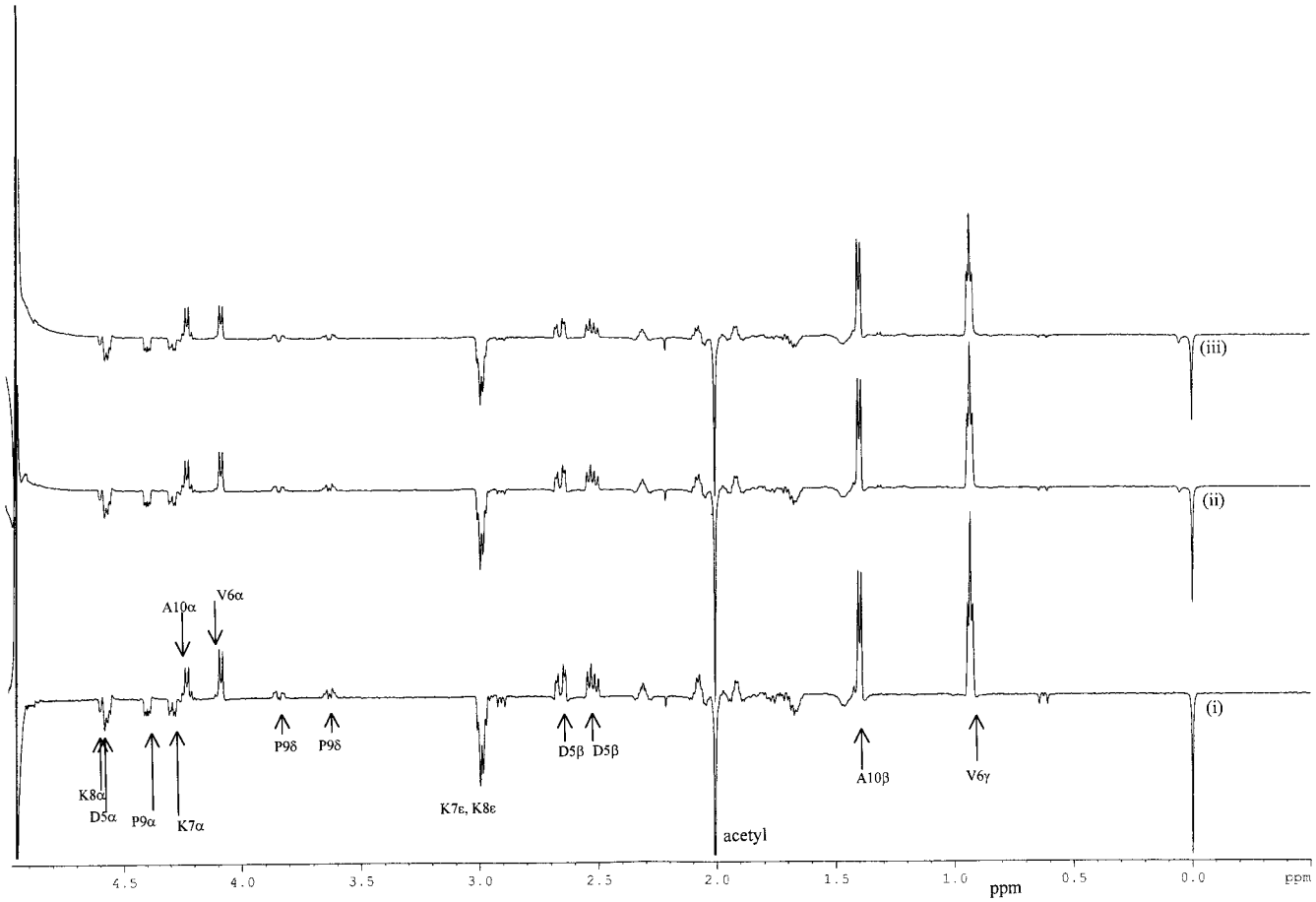

FIG. 3. **Interaction of A1-type ELC peptide 6–10 with F-actin determined by ^{1}H NMR.** CPMG spectra of residues 6–10 from rabbit skeletal Al-type ELC (*N*-acetyl-DVKKPA-$CONH_2$) in 5 mM phosphate buffer, pH 7.2, containing 2 mM $MgCl_2$, 0.3 mM NaN_3, 0.1 mM DSS at 285 K. This peptide was α-*N*-acetylated to mimic its position in the protein. Peptide concentration was maintained at 500 μM in all experiments. *i*, peptide alone; *ii*, peptide plus 33.3 μM F-actin (ratio 15:1); *iii*, peptide plus 50 μM F-actin (ratio 10:1).

17, 30), leading to the original suggestion that it might contact actin directly (30).

Interaction of N-terminal Peptides from A1-type ELC with Actin Determined by ^{1}H NMR—The CPMG spectra of the N-terminal 10 residues of rabbit skeletal Al-type ELC is shown in Fig. 1. The peaks were assigned as described in the Experimental Procedures. Broadening of specific peptide resonances was induced by incremental additions of F-actin and can be attributed to a reduction in their T_2 relaxation time. This not only leads to a broadening of peaks but also to a reduction in peak amplitude in CPMG spectra (which are after all a 'measure' of T_2). Exchange between the free and bound peptide species allows us to observe a weighted mean of the T_2 for the two species in the free peptide. Those residues involved in binding (*i.e.* are immobilized) have a much shorter T_2 in the bound state and hence in the observed state.

The residues most notably broadened occurred at the N terminus of the 10 residue peptide: Ala^1 (β-CH_3 and α-CH, whereas Ala^{10} resonances are largely unaffected), Pro^2 (α-CH and δ-CH_2, note Pro^9 α-CH is largely unaffected), ε-CH_2 of Lys (from these data it cannot be determined which Lys are most affected) and, to a lesser extent, resonances arising from Asp^5 and Val^6 (Fig. 1). The interaction between the peptide and actin is largely ionic since addition of 100 mM KCl to the complex considerably weakens the interaction and the spectrum resembles that of the peptide alone. These data (and those in Fig. 3) confirm earlier observations (31) that trimethylation is not prerequisite for actin binding.

A clearer idea of which residues in the peptide were contributing to complex formation with actin was found by comparing the binding of a peptide containing residues 1–6 with one containing residues 5–10 (Figs. 2 and 3). The peptide 1–6 clearly bound to actin with resonances arising from Ala^1, Pro^2, Lys^3 and Lys^4 being the most significantly broadened. Asp^5 and Val^6 were hardly affected. Binding to actin appeared to be weaker with the shorter peptide since a higher concentration of actin was required to achieve significant broadening (Fig. 2). On the other hand, titration of peptide 5–10 with the same range of actin concentrations did not reveal that any significant interaction was occurring (Fig. 3). This result was interesting in two respects. Firstly, it showed that Lys ^{3} and Lys^4 were involved in the interaction with actin rather than Lys^7 and Lys^8. Secondly, since the two shorter peptides are virtually 'palindromic' (and certainly contain the same number of charges), the data in Fig. 3 acted as good controls for those described in Figs. 1 and 2. Further confirmation of this is shown in Fig. 4. In this two-dimensional TOCSY, the signals of the 4 Lys residues in the larger peptide are well resolved. Addition of F-actin causes those cross-peaks arising from Lys^3 and Lys^4 to be considerably reduced in intensity, without anywhere near as much effect on Lys^7 and Lys^8. The TOCSY also shows reduced T_2 for the 'immobilized' residues, *i.e.* those which disappear.

The Size of the Side Chain at Position 1 Is Unimportant for Actin Binding—Since the CH_3 of Ala^1 was found to selectively broaden on addition of actin, whether or not the α-N terminus was trimethylated (above and 28), the role of this side chain was investigated using the HmAtELC(A1V) mutant. When hybridized into rabbit skeletal S1, the HmAtELC(A1V) mutant

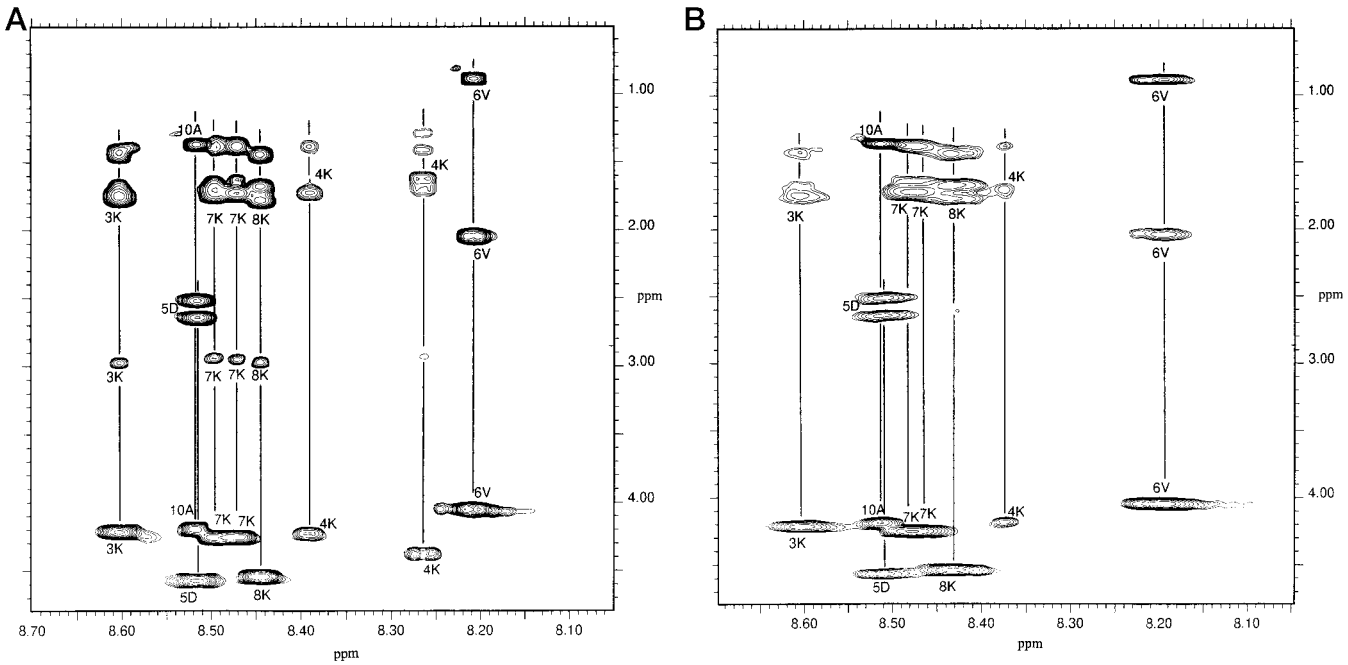

FIG. 4. **^{1}H two-dimensional TOCSY spectra of A1-type ELC peptide 1–10 in the absence and presence of F-actin.** The peptide concentration in each panel was 3.5 mM in 5 mM phosphate buffer, pH 6.8, containing 42 mM KCl, 2 mM $MgCl_2$, 0.3 mM NaN_3. *A*, peptide alone; *B*, peptide plus 325 μM actin. The region illustrated shows correlations between the backbone amide protons (*horizontal axis*, between 8.1 and 8.7 ppm) and the aliphatic side chain protons (*vertical axis*, between 0.5 and 5.5 ppm).

TABLE I
Actin-activated MgATPase kinetic constants for hybrid S1s formed between rabbit skeletal S1 and various mutant ELCs

Hybrid	K_M for actin[a]	k_{cat}[a]	n[b]
	μM	s^{-1}	
S1(A1)[c]	4.2 ± 0.6	13.7 ± 1.1	20
S1(A2)[c]	31.9 ± 9.9	34.6 ± 9.5	24
S1(HmAtELC)	5.4 ± 1.5	15.3 ± 2.8	32
S1(HmAtELC(A1V))	5.2 ± 0.8	15.7 ± 1.5	24
S1(HmAtELC(K4R))	3.5 ± 0.4	14.3 ± 0.9	22
S1(HmAtELC(K4A))	10.8 ± 1.6	20.6 ± 2.3	24
S1(HmAtELC(K4D))	22.8 ± 6.7	28.3 ± 7.0	24
S1(HmAtELC(K3A))	9.4 ± 0.9	15.7 ± 1.1	22

[a] Values are shown ± standard error.
[b] Number of separate determinations.
[c] Values for rabbit skeletal S1(A1) and S1(A2) are taken from Ref. 11.

gave actin-activated MgATPase kinetic parameters which were identical to the wild-type (Table I, Fig. 5A). Furthermore, this mutant light chain (both free and hybridized into S1) cross-links to F-actin (Fig. 5B). The amount of HmAtELC(A1V)-actin product produced is similar to the amount of wild-type HmAtELC product under the same conditions (see also 11, 13). Thus, we conclude that the size of the side chain is unimportant in kinetic modulation and actin binding.

A Positive Charge at Positions 3 and 4 Is Essential for Full Kinetic Modulation—A series of mutants were prepared in which the positive charge at position 4 was varied from positive (wild-type HmAtELC and HmAtELC(K4R)), through neutral (HmAtELC(K4A)) to negative (HmAtELC(K4D)). When these were hybridized into rabbit skeletal S1, the kinetic parameters of the hybrids became progressively more S1A2-like (Table I, Fig. 6). Interestingly, the results for HmAtELC(K4R) hybrid showed a lower apparent K_m for actin than the wild-type, suggesting that this ELC may even have a higher affinity for actin. Abolition of the positive charge at position 3 (HmAtELC(K3A)) gives very similar results to the same change at position 4 (Table I, Fig. 6).

All four mutant ELCs can be cross-linked to F-actin (Fig. 7). However, there are clear differences in the amount of cross-linked product produced: HmAtELC(K4R) cross-linked to the same extent as wild-type HmAtELC but to a greater extent than HmAtELC(K4A), which in turn cross-linked better than HmAtELC(K4D). HmAtELC(K4A) and HmAtELC(K3A) cross-linked to similar extents. The results with S1 hybrids of these mutants are similar (not shown). It should be emphasized that cross-linking does not report on the equilibrium situation but rather it captures equilibrium complexes once formed and prevents them from dissociating; depending on the binding constant, the efficiency of the cross-linking reaction and experimental variables. Nevertheless, the results in Fig. 7 are qualitatively in agreement with the apparent K_m values derived from kinetic analysis which suggest that the mutant ELCs bind actin in the following order of affinity: HmAtELC(K4R) > HmAtELC(K4A) ≈ HmAtELC(K3A) > HmAtELC(K4D).

The Apparent K_m for Actin Controls k_{cat}—If the apparent K_m for actin is plotted against k_{cat} for all these mutants then a clear correlation is seen (Fig. 8). The correlation is significant at the 1% level (r^2 = 0.98, degrees of freedom = 6). This suggests that the K_m controls k_{cat} up to a limit defined by S1(A2). This strengthens our belief that actin binding (reflected by K_m) by the wild-type A1-type ELC results directly in kinetic modulation (reflected by k_{cat}): if the first is weakened, so is the second.

DISCUSSION

The NMR data strongly suggest that the major interaction between F-actin and the A1-type ELC occurs mainly in the N-terminal APKK . . . sequence. The slight broadening of the Asp[5] and Val[6] signals in Fig. 1 and Fig. 2, which do not occur in Fig. 3, probably reflect the fact that the peptide is being immobilized by binding to actin directly N-terminal to them, restricting their motion and so broadening their resonances. It should also be noted that the N-terminal sequences of all vertebrate striated muscle Al-type ELC so far sequenced (see Swiss Protein Data base) begin with either the sequence APKK. . . . or PPKK . . . It was surprising that the A1V mutant

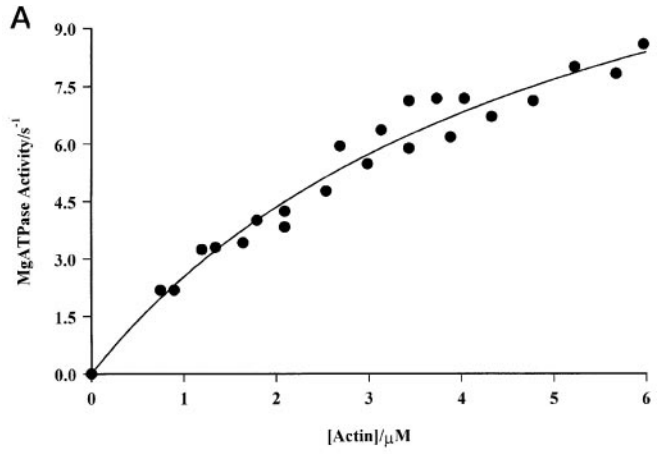

FIG. 5. **Kinetic properties of the mutant S1 (HmAtELC(A1V)) hybrid and its cross-linking to F-actin.** *A*, plot of MgATPase activity against F-actin concentration for mutant S1(HmAtELC(A1V)). The line was fitted to the data by non-linear curve fitting as described under "Experimental Procedures." *B*, SDS-PAGE of the products formed from EDC cross-linking of F-actin to HmAtELC or HmAtELC(A1V) alone or hybridized into S1. *Lanes 2–6* and *8* are cross-linked proteins. *Lane 1*, S1 (HmAtELC) (10 μM); *lane 2*, S1(HmAtELC) (10 μM); *lane 3*, S1(HmAtELC) (4 μM) plus F-actin (20 μM); *lane 4*, S1(HmAtELC(A1V) (4 μM) plus F-actin (20 μM); *lane 5*, HmAtELC (30 μM) plus F-actin (10 μM); *lane 6*, HmAtELC(A1V)) (30 μM) plus F-actin (10 μM); *lane 7*, F-actin (10 μM); *lane 8*, F-actin (10 μM); *lane 9*, HmAtELC (15 μM). 10-μl samples were loaded onto the gel.

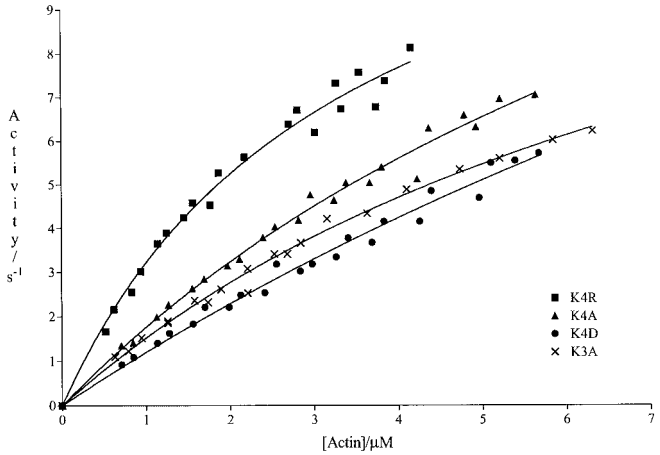

FIG. 6. **Plot of MgATPase activity against F-actin concentration for various mutant ELC S1 hybrids.** Kinetic constants were determined by non-linear curve fitting as described under "Experimental Procedures." A series of HmAtELC mutants were prepared in which the positive charge at position 4 was varied from positive to neutral or negative and that at position 3 was made neutral. The mutant light chains were hybridized into S1. *Squares*, S1(HmAtELC(K4R)); *triangles*, S1(HmAtELC)(K4A)); *circles*, S1(HmAtELC(K4D)); *crosses*, S1(HmAtELC)(K3A)).

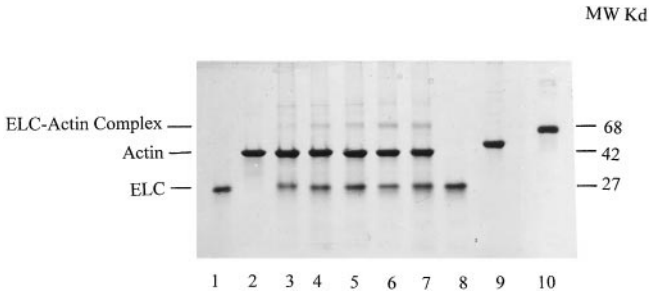

FIG. 7. **Cross-linking of various HmAtELC mutants to F-actin analyzed by SDS-PAGE.** *Lanes 3–9* are EDC-cross-linked proteins. *Lanes 3–7* contain 30 μM light chain and 10 μM F-actin. *Lane 1*, HmAtELC (15 μM); *lane 2*, F-actin (10 μM); *lane 3*, HmAtELC(K4D) plus F-actin; *lane 4*, HmAtELC(K3A) plus F-actin; *lane 5*, HmAtELC(K4A) plus F-actin; *lane 6*, HmAtELC (K4R) + F-actin; *lane 7*, HmAtELC + F-actin; *lane 8*, HmAt ELC (15 μM); *lane 9*, F-actin (10 μM); *lane 10*, BSA. 10-μl samples were applied to each lane.

had no effect on the ELC-actin interaction, given the results in Fig. 1 and Fig. 2. The most plausible explanation for this is that the side chain itself is not involved in the contact with actin and thus increasing its size does not interfere with the interaction. However, the NMR data suggested that some feature of Ala[1] was important for binding to actin. It could be that all that is required for actin interaction is a positive charge on the N terminus, which will be provided by both the trimethylation in the native light chain or a free amino terminus in the peptides. Some support for this comes from our observation (11) that it was impossible to detect a free N-terminal group in the actin/recombinant HmAtELC cross-linked adduct. (Note that the native actin N-terminal amino acid is acetylated.) This would explain the selective broadening of the *N*-trimethyl groups and the Ala[1] methyl in the NMR spectra as this residue takes on the correlation time of the actin polymer.

It is worth commenting further on the possible significance of the N-terminal trimethylation. The addition of three methyl groups to the N terminus must be an energetically costly exercise for the organism, and we would, therefore, expect it to serve some purpose *in vivo*. We suggest two possible explanations for this paradox. The measurements we make are steady state ones in order to determine the apparent K_m for actin and the k_{cat} for MgATP turnover. It may be that the main influence of the trimethylation is on the initial pre-steady state steps in

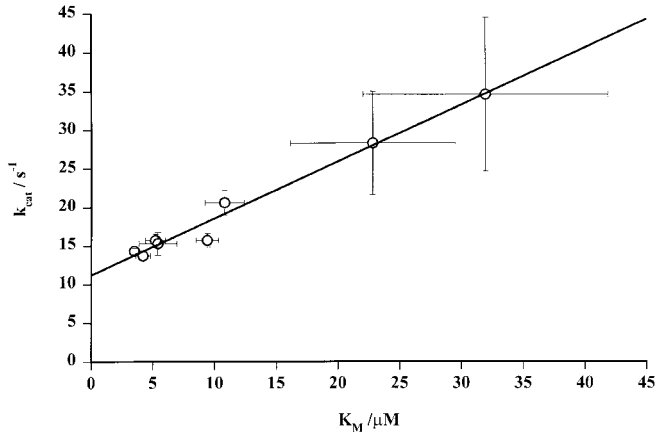

FIG. 8. **Correlation between k_{cat} for MgATP turnover and the apparent K_m for actin.** The values shown are those taken from Table I derived by non-linear curve fitting, and the *error bars* are the standard errors obtained in this process. The *line* is the linear regression line as calculated by Fig.-P for Windows.

the pathway whose influence is not reflected significantly in K_m and k_{cat}. Alternatively, as we have suggested above, it could be that trimethylation or a free amino group contribute approxi-

mately equally to actin binding but that the function of the post-translational modification is to protect the N terminus of the ELC from degradation within the cell (32). Trimethylation may have been selected over other alternative (less energetically costly) N-terminal modifications (such as acetylation) as it retains a positive charge at the N terminus. It is interesting to note in this context that A2-like ELCs (which do not interact with actin (16)) are N-terminally acetylated (as are all other myofibrillar proteins). It is clear, however, that a positive charge at both positions 3 and 4 is required for full actin binding and kinetic modulation. Indeed it is possible that actin binding requires merely a concentration of positive charge in the correct orientation in the N-terminal region with contributions from the N terminus and $Lys^{3,4}$, with Pro^{2} providing the scaffold.

The results are in agreement with the recent work of Sweeney (33), which showed that the mutation of the equivalent Lys residues in rabbit fast skeletal muscle A1-type ELC resulted in increased shortening velocities when the ELCs were reconstituted into permeabilized muscle fibers. This is entirely consistent with our data which shows that the equivalent changes in our system result in increased maximal MgATP turnover. Furthermore, by showing that these mutations also resulted in decreased affinity for actin by the ELC (as reflected in the increased K_m values and the cross-linking data) we have established a direct, causal relationship between actin binding and the modulation of the kinetics of S1.

Recent work from the laboratory of Solaro and co-workers (34) further suggest a role for the A1-type ELC N-terminal region in the regulation of contractile activity, at least in cardiac muscle. These workers found that low concentrations of a peptide from the N-terminal region of ventricular A1-type ELC induced a supramaximal increase in MgATPase activity only at submaximal Ca^{2+} levels when added to rat cardiac myofibrils. This effect required a full complement of thin filament regulatory proteins and occurred in a highly cooperative manner that could involve activation of the whole thin filament at low peptide to thin filament ratios.

We have established in this and previous work (11, 13) the importance of the N-terminal residues of A1-type ELCs in modulating myosin motor function. This modulation results directly from these residues binding to actin and these two events are tightly coupled. What remains to be discovered is how this actin binding event some 8 to 10 nm from the active site of myosin (as judged from the models of acto-S1 derived from the crystal structures of the proteins (35–37)) results in kinetic modulation. In this context, the results with HmAtELC(K4D) are particularly interesting. Although the light chain cross-links to actin (but to a lesser extent than the other mutants and the wild-type), its k_{cat} is essentially S1(A2)-like. This suggests that the free energy of binding resulting from the ELC-actin interaction is used to drive conformational changes which influence the active site. The much weakened interaction in the case of HmAtELC(K4D) is insufficient to drive this process. In the case of HmAtELC(K3A) and HmAtELC(K4A), the binding energy is only sufficient to drive the process part-way. Communication between the light chain binding domain and the motor domain is a common feature of all muscle myosin types (1–4, 7–10); it remains to be seen if the pathways of communication between the light chains and the active site also have features in common.

Acknowledgments—We thank Sue Brewer and Nina Sewell for their skilful technical assistance and Tony Pemberton for maintenance of the NMR facility.

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