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Modifying preselected sites on proteins: The stretch of residues 633–642 of the myosin heavy chain is part of the actin-binding site

(peptide/ATPase/subfragment-1/fluorescence/complementariness)

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ABSTRACT **We have designed an "antipeptide" capable of firmly and specifically interacting with a preselected stretch of myosin S-1 heavy chain. Covalent attachment of this antipeptide to its target stretch, residues 633–642, does not affect the intrinsic ATPase activities of the protein but significantly reduces the actin-binding capabilities of the myosin head.**

This work had a dual purpose. We wished to test the possibility of designing a peptide that would attach firmly and specifically to a selected stretch of a protein sequence (an "antipeptide," by analogy with antibody). Were this objective to be attained, we wished to use antipeptides to probe stretches of the myosin sequence suspected of having specific functions in contraction. The first design was intended to bind to the stretch of residues 633–642 of the myosin subfragment 1 (S-1) sequence consisting of lysines interspersed with glycines; so it consisted of aspartates with corresponding interspersals, thus providing a number (five) of attractive coulombic interactions that contributed to stability. The stretch and the antipeptide were also long enough for specificity-demanding complexity to be expressed. The Chou–Fasman heuristic (1) predicted little structure for the target stretch, so we did not anticipate that much binding energy would be spent in unwinding the stretch.

Our design included an extra cysteine to accommodate labels. Since several amino–carboxyl interactions were anticipated, the antipeptide had the potential of being permanently bonded through carbodiimide crosslinks. Previous work (2–4) has suggested that actin binds at or near this target stretch. Additionally, the presence of a consensus sequence, Gly-Xaa-Xaa-Xaa-Xaa-Gly-Lys, known to be a phosphoryl subsite in protein kinases (5) and recent experiments on soluble fragments of S-1 heavy chain (6) have suggested that the polyphosphate chain of ATP might interact with this stretch. But actin and nucleotide are thought to bind at other stretches as well, so the functional consequences of binding this stretch only could not have been foreseen.

MATERIALS AND METHODS

Chemicals. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin and α-chymotrypsin were from Worthington. Endoproteinase ArgC was from Boehringer Mannheim. CNBr, 1-ethyl-3[-3(dimethylamino)propyl]carbodiimide (EDC), and EDTA were from Sigma. *N*-(7-Dimethylamino-4-methylcoumarinyl)maleimide (DACM) was from Polyscience (Worthington, PA). 1-Hydroxypyrene-3,6,8-trisulfonate (HPTS) was from Molecular Probes. All other chemicals were of the highest analytical grade.

Preparation of Actin and S-1 Derivatives. Rabbit skeletal muscle actin and myosin were prepared as in refs. 7 and 8, respectively. S-1 was obtained by digestion of myosin filaments with α-chymotrypsin (9) and was further purified by HPLC with a 535 CM-TSK column (LKB), equilibrated with a solution of 10 mM sodium phosphate 0.1 mM EDTA, and 0.1 mM sodium azide (pH 7.0). Separated or mixed isoenzymes of S-1 with alkali light chain 1 [S-1(A_1)] and 2 [S-1(A_2)] were then dialyzed against a solution of 10 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes) and 0.1 mM sodium azide (pH 7.0). S-1 was cleaved by trypsin with a trypsin/S-1 weight ratio of 1:100, at 25°C, for 30 min in 50 mM Tris·HCl (pH 8.0) (10). Proteolysis of S-1 heavy chain by endoproteinase ArgC was performed in 50 mM Tris·HCl (pH 8.0) containing 15 units of ArgC protease per mg of protein, for 120 min at 25°C (11). Both proteolytic reactions were terminated by incubating the samples for 5 min in 3 vol of a boiling solution of 50 mM Tris·HCl, 2% (wt/vol) $NaDodSO_4$, 1% 2-mercaptoethanol, and 50% (vol/vol) glycerol (pH 8.0). CNBr cleavage of native S-1 or antipeptide S-1 complexes was performed as in ref. 12.

Antipeptide Synthesis and Modification. The antipeptide Cys-Gly-Gly-Asp-Asp-Gly-Gly-Asp-Asp-Asp-Gly was synthesized (by using the AB1 procedure on a model AB1430 peptide synthesizer) and supplied to us by the University of California, San Francisco, Biomolecular Resources Facility. Crude antipeptide (0.5–1 mM) was modified with either iodoacetamide or DACM by using a 1.5-fold excess of reagent in 10 mM Hepes (pH 8.0) at 25°C for 14 hr. Unmodified and modified antipeptide were further purified by HPLC with a Hi-pore RP318 reverse-phase column (LKB) with a gradient from 0 to 90% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid. Concentration of unmodified and iodoacetamide-modified antipeptide was obtained by amino acid analysis (by using the ninhydrin method in a model 121 MB-8167 Beckman analyzer). Concentration of DACM-antipeptide was obtained by using a 1:1 stoichiometry and an $\varepsilon_{380} = 1.98 \times 10^4\ M^{-1}\cdot cm^{-1}$ for DACM.

Binding Experiments. When antipeptide binds to S-1 in a very slightly buffered system, there are net proton releases that we detected as small pH changes; these changes were amplified by a very sensitive fluorescence pH indicator, HPTS (18). Calibration was accomplished by adding aliquots of 2 mM HCl to a solution containing 15 μM S-1, 0.5 mM HPTS, and 0.1 mM Tes (pH 8.00) in the absence or presence of 50 mM KCl. Decrease of fluorescence was a linear function of pH decrease from pH 8.00 to 6.90; the slope was 6.66% per 0.1 pH unit in the absence as well as in the presence of 50 mM KCl. The binding assay was performed by adding increasing amounts of antipeptide or iodoacetamide-antipeptide (dissolved in 0.1 mM Tes) to a solution containing 15 μM S-1, 0.5 μM HPTS, and 0.1 mM Tes (pH 7.50) in the absence or presence of various KCl concentrations. Fluorescence emis-

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Abbreviations: S-1, subfragment 1; S-1(A_1), isoenzyme of S-1 with alkali light chain 1; S-1(A_2), isoenzyme of S-1 with alkali light chain 2; EDC, 1-ethyl-3[-3(dimethylamino)propyl]carbodiimide; HPTS, 1-hydroxypyrene-3,6,8-trisulfonate; DACM, *N*-(7-dimethylamino-4-methylcoumarinyl)maleimide.

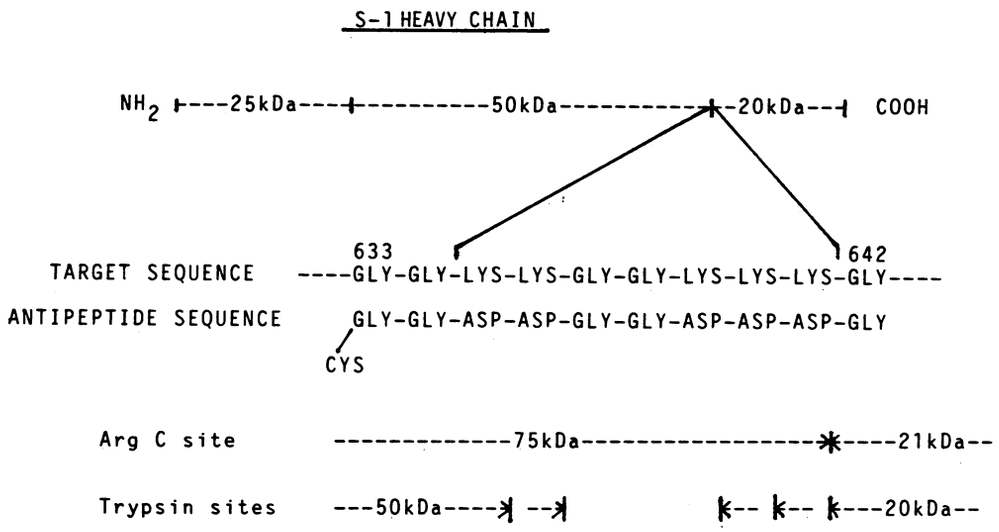

FIG. 1. Sequence of the antipeptide and of the target stretch residues 633–642 of skeletal muscle myosin S-1 heavy chain.

sion changes at 520 nm (λ_{excit} = 454 nm) were monitored with an SLM model 8000 fluorometer, maintained at 20°C. Prior to each experiment, S-1 was extensively dialyzed against 0.1 mM Tes (pH 7.50), sedimented for 60 min at 160,000 × g, and filtered through a Millipore membrane (pore size, 0.45 μm).

The association of actin with S-1(A_2) or antipeptide-S-1(A_2) complexes was studied by cosedimentation in a solution of 50 mM Tris·HCl and 2.5 mM $MgCl_2$ (pH 7.5), in the absence or presence of 4 mM ADP or 2.5 mM ATP (13).

Crosslinking Reactions. Various proportions of unmodified or modified antipeptide were crosslinked to S-1(A_1) or S-1(A_2) by 10 mM EDC in 10 mM Tes (pH 7.0) at 20°C for 60 min. The reactions were quenched by 100 mM 2-mercaptoethanol. Crosslinked derivatives were then concentrated by ammonium sulfate precipitation and purified by Sephadex G-100 chromatography (1.5 × 20 cm column) equilibrated with a solution of 10 mM Tes and 0.1 mM sodium azide (pH 7.0). Antipeptide-S-1 complexes were further purified on a DE-52 column (1.5 × 5 cm) equilibrated with a solution of 10 mM Tes and 0.1 mM sodium azide (pH 7.0) (antipeptide-S-1 derivatives were eluted at 50 mM NaCl). By using a 4-fold excess of antipeptide during the crosslinking reaction, we could purify 50% of the initial S-1(A_2) containing 1.10 antipeptides per S-1 (the stoichiometry was measured by using the DACM-antipeptide-S-1 complex).* In all experiments, antipeptide-S-1 complexes were compared to the control, EDC-treated S-1 (identical procedures were used but the antipeptide was omitted).

Protein Concentrations. Protein concentrations were optically measured assuming A_{280} = 7.5 for native S-1 and A_{280} = 11 for actin. The concentrations of antipeptide-S-1 complexes were measured by the Bradford assay with native S-1 as a standard (14).

NaDodSO$_4$/PAGE. Polyacrylamide gel electrophoresis was as described (15).

S-1 ATPase. Ca^{2+}- and K^+/EDTA-dependent ATPase activities and actin-activated Mg-ATPase activity were measured at 25°C, as described (10). Mg^{2+}-ATPase dependence was measured by the method of Mandelkow and Mandelkow (16). Conditions were 1 μM enzyme, 50 mM Tris·HCl, 2.5 mM $MgCl_2$, and 10 mM KCl (pH 8.0) at 25°C.

*Because EDC treatment of S-1(A_1) isoenzyme at low ionic strength produces a significant amount of heavy chain-light chain covalent complex, most of the experiments were performed with the S-1(A_2) isoenzyme.

RESULTS AND DISCUSSION

The sequence of the antipeptide directed against the stretch between residues 633 and 642 is in Fig. 1. We reached this design on the basis of very simple considerations. (*i*) We placed a negatively charged residue of the antipeptide opposite to each positively charged residue of the stretch, and we placed another neutral residue (glycine) opposite to each neutral residue (glycine). (*ii*) The Chou–Fasman heuristic (1) indicated that the target stretch (and also the antipeptide) was probably unstructured.

Interaction of Antipeptide with S-1. By using the high sensitivity of the HPTS fluorescence to pH, we could quantify the increase of [H^+] resulting from the antipeptide–S-1 interaction, and we, therefore, could estimate the strength of this interaction. Fig. 2 shows the decrease in fluorescence observed when increasing concentrations of antipeptide were added to a solution containing 15 μM S-1 at very low ionic strength (μ < 0.1 mM). The data were fitted to the equation $K_a = [B]/\{([S\text{-}1] - [B]) + ([\text{antipeptide}] - [B])\}$, where K_a is the association constant, [B] is the concentration of antipeptide bound to S-1 $\{[B] = [S\text{-}1](F_0 - F)/(F_0 - F_{max})$, where F, F_0, and F_{max} are fluorescence with the antipeptide, fluorescence without the antipeptide, and maximal fluorescence}, and [S-1] and [antipeptide] are the total concentrations of S-1 and antipeptide, respectively. This equation gave $K_a = 2.1 \times 10^6\ M^{-1}$, assuming an antipeptide/S-1 stoichiometry of 1:1.

By using the same method, we studied the binding of antipeptide as a function of ionic strength, from 0.002 to 0.050 M. The data (Fig. 2 *Inset*) show that, at least qualitatively, the binding of antipeptide to S-1 follows the simple linear Debye–Huckel relationship, log K_a vs. $\mu^{1/2}$, thus indicating that the interaction is mostly coulombic.

Irreversible Attachment of Antipeptide to Its Target Stretch. Covalent attachment of antipeptide to S-1 heavy chain was accomplished by EDC crosslinking. Thus, by using a low-ionic-strength buffer (10 mM Tes), it has been possible to crosslink unmodified as well as DACM-modified antipeptide to S-1 and to purify an antipeptide-S-1 complex containing a 1.1:1 molar ratio of antipeptide/S-1.

Localization of the antipeptide bound along the S-1 heavy chain was achieved by proteolytic and chemical degradation of the DACM-antipeptide-S-1(A_2) complex. Inspection of the gel electrophoretic pattern of such an experiment clearly shows that only S-1 heavy chain is labeled by the DACM-carrying antipeptide (Fig. 3, lane d). After ArgC proteolysis,

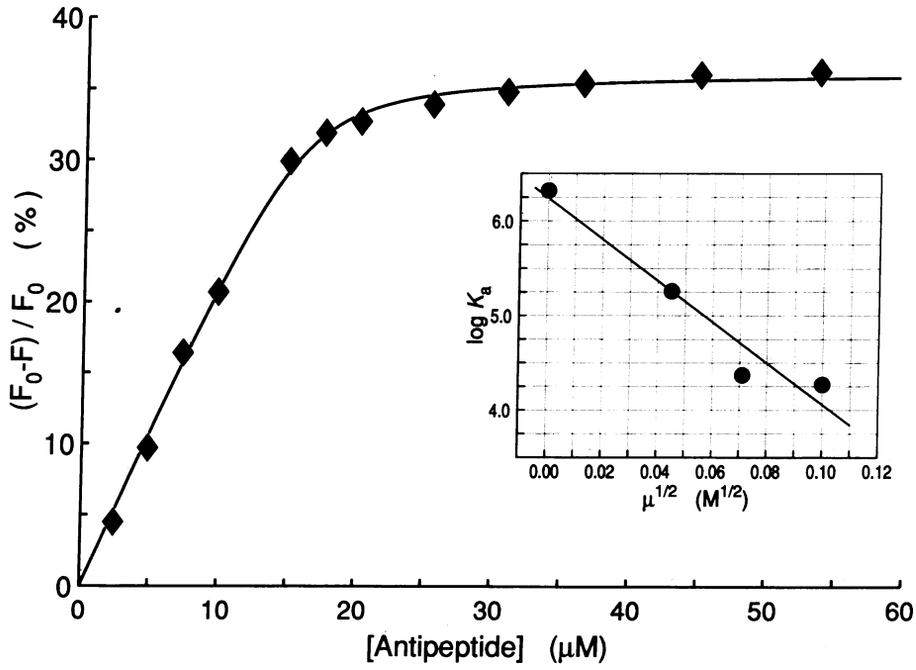

FIG. 2. Binding of antipeptide to S-1 as monitored by fluorescence. F and F_0 represent the fluorescence in the presence and in the absence of antipeptide. (*Inset*) Dependence of binding constant on ionic strength.

only 18% of the heavy chain is cleaved (lane e) compared to 90% of control EDC-treated S-1 (lane b); this result shows that antipeptides were on at least 82% of the heavy chains thus protecting against the unique ArgC cleavage (Fig. 1; R. Kassab, personal communication) and, therefore, were on the target sequence. After trypsin degradation (lane f), only the 50-kDa and 20-kDa fragments carry some fluorescence. This residual fluorescence can be explained as a heterogeneity in the trypsin degradation of the 50-kDa to 20-kDa junction. Finally (lane g), after CNBr cleavage, there is only one major fluorescent band carrying 85% of the total fluorescence.

By knowing that under our experimental conditions, 85% of the target sequence bore crosslinked antipeptide, we first studied the effect of blocking the stretch between residues 633 and 642 on the intrinsic S-1 ATPase activities. As shown in Table 1, if we compare the Ca^{2+}-, K^+-, and Mg^{2+}-ATPase activities of the antipeptide-S-1 complex to the same activities of control EDC-treated S-1, none of them is significantly altered by the covalent interaction of the antipeptide with its target sequence. However, we must note that under the low-ionic-strength conditions used, EDC treatment of S-1 slightly and specifically affects all of these ATPase activities.

Unlike unaffected Mg^{2+}-ATPase activity, actin-activated Mg^{2+}-ATPase activity is strongly inhibited when antipeptide is bound to its target sequence. A double reciprocal plot (Fig. 4) shows that the loss of actin activation is restored at high actin concentrations. This behavior is most naturally explained by assuming that, in the active state, the presence of bound antipeptide on the target stretch has decreased but not abolished the affinity of the S-1 for actin. ArgC is known to cleave within the target stretch, so covalent bonding of the antipeptide should protect against ArgC—i.e., the more the protection, the more the stretches are bonded to antipeptides. The experiments above suggest that bonding of antipeptide reduces the actin-activated activity. Therefore, actin-activated activity should be a descending linear function of protection (i.e., uncleaved chain); this is what we observe (Fig. 5). So, this result too shows that the antipeptide reached the right target.

Finally, on comparing the actin binding constants of control S-1(A_2) and of the antipeptide-S-1(A_2) complex, it appears that the presence of antipeptide significantly decreases the affinity constants of actin to S-1 in the absence as well as in the presence of ADP·Mg^{2+} or ATP·Mg^{2+} (Table 2).

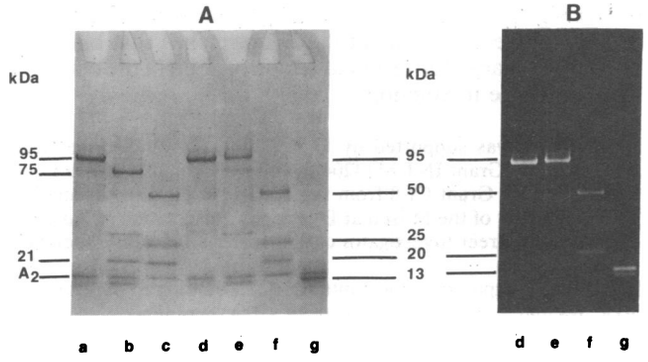

FIG. 3. Representative $NaDodSO_4$/polyacrylamide gel showing proteolytic degradation and chemical cleavage of control EDC-treated S-1 and DACM-antipeptide-S-1 complex. Control S-1 (lane a) or DACM-antipeptide-S-1 (lanes d) was digested by ArgC (lanes b and e) or trypsin (lanes c and f) and DACM-antipeptide-S-1 was cleaved by CNBr (lanes g). The gel was stained with Coomassie blue (*A*) or viewed under ultraviolet light (*B*).

CONCLUSION

Like its aims, the conclusions of this paper are in two categories. The aim of designing an antipeptide that seeks out

Table 1. ATPase activities of native and modified S-1(A_2)

S-1 form	ATPase activity, s^{-1}		
	Ca^{2+}-ATPase	K^+/EDTA-ATPase	Mg^{2+}-ATPase
Native S-1	1.6	7.8	0.045
EDC-treated S-1	3.9 (2.4)	5.6 (0.72)	0.157 (3.5)
Antipeptide-S-1	3.8 (2.4)	5.6 (0.72)	0.170 (3.8)

Numbers in parentheses are ratios of modified S-1 over native S-1.

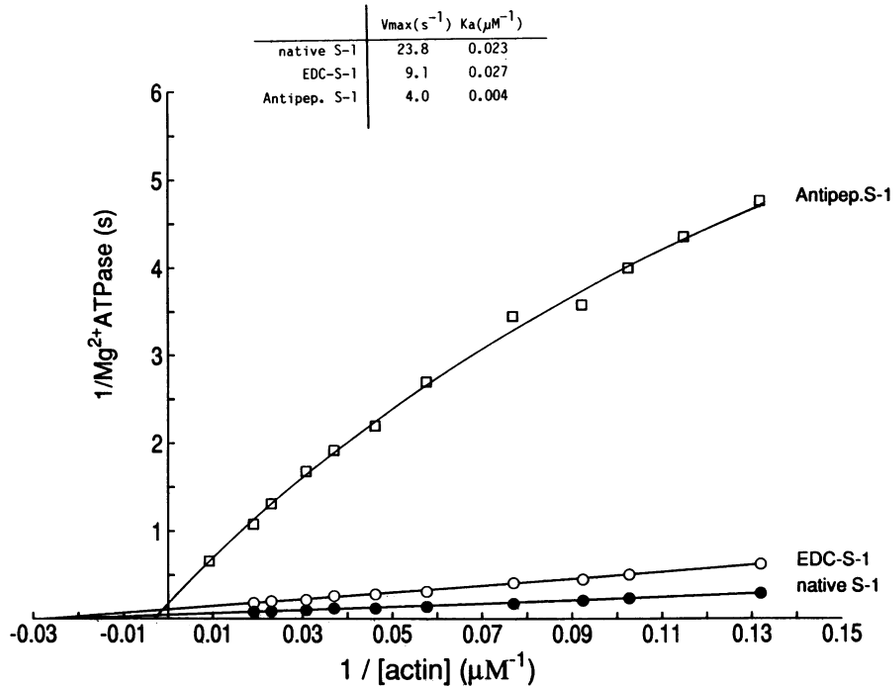

FIG. 4. Double reciprocal plot of Mg^{2+}-ATPase activity vs. filamentous actin concentration. Parameters V_{max} and K_a were obtained by fitting data on EDC-treated S-1 and native S-1 to linear functions and were obtained graphically for the antipeptide-S-1 complex.

a selected stretch of a protein sequence and binds to it with significant affinity and specificity seems to have been achieved in this case. Since it is natural to wonder whether this is a procedure of wide applicability, we should note that probably the procedure succeeded in this case because of several felicitous circumstances: the protein–antipeptide attraction was obviously coulombic; this made it easy to choose the nature and position of the residues in the design, and it offered the advantage of permanent bonding with EDC. The stretch (and, therefore, the antipeptide) was probably unstructured, so little of the binding energy was wasted in unwinding structure. Finally, the stretch selected was long enough (contained enough information) to allow high specificity to be built into the antipeptide.

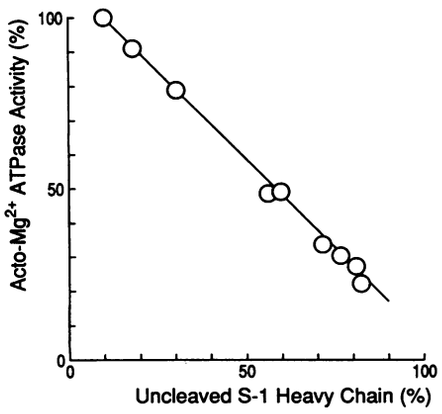

FIG. 5. Actin-activated Mg^{2+}-ATPase activity of S-1 related to antipeptide-protected fraction of S-1. Mg^{2+}-ATPase activity was measured in the presence of 20 μM actin for antipeptide-S-1 complexes obtained by crosslinking experiments at various antipeptide concentrations. Purified antipeptide-S-1 complexes were then degraded by ArgC, which cleaves the peptide bond between residues 641 and 642 only in the absence of protector antipeptide. The percentage of uncleaved S-1 heavy chain was obtained by densitometry of the gel electrophoretogram.

The study of structure–function in S-1 by using the antipeptide was rewarded by several findings. *A priori*, the stretch between residues 633 and 642, being a cluster of positive charges, might have been a polyphosphate binding site, but this turned out not to be the case because even permanently bonded antipeptide has a negligible effect on the intrinsic ATPases of control S-1.

On the other hand, S-1-bound antipeptide clearly inhibited the binding of actin to S-1, thus showing directly what earlier experiments (2–4) had shown indirectly; that the sequence from residue 633 to residue 642 is a part of a composite actin binding site on S-1. Since increasing actin concentration restored actin activation of S-1 ATPase and since antipeptide binding reduces, but does not abolish, the affinity of S-1 for actin, other parts of S-1 heavy chain are certainly involved in actin binding.

Finally, Table 2 clearly shows that antipeptide binding at residues 633–642 destabilizes the rigor, as well as the active, acto–S-1 complexes. Combination of our present result with earlier works (2, 17) indicates that the stretch from residue 633 to residue 642 is one of the S-1 stretches that binds the negatively charged N-terminal region of actin in the course of the contractile interaction.

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Table 2. Comparative constants for actin binding to control EDC-treated S-1(A_2) and antipeptide-S-1$(A)_2$ complex

Addition	K_a, M^{-1} (no. × 10^{-6})	
	EDC-treated S-1	Antipeptide-S-1
Nothing (rigor)	25.0	1.9
+ ADP·Mg^{2+}	1.9	0.13
+ ATP·Mg^{2+}	0.022	0.004

Values were determined from Scatchard plots obtained from binding experiments.

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