# Role of Charged Amino Acid Pairs in Subdomain-1 of Actin in Interactions with Myosin†

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ABSTRACT: Yeast actin mutants with alanines replacing charged amino acid pairs D24/D25, E99/E100, D80/D81, and E83/K84 were studied to assess their role in interactions with myosin. In a previous report *Dictyostelium* actin filaments with residues D24/D25 or E99/E100 replaced with histidines showed complete or partial loss of filament sliding in the *in vitro* motility assay [Johara, M., *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 2127−2131]. In the motility experiments reported here, actin filaments with alanines substituted at D24/D25 or E99/E100 moved in the presence of 0.7% methylcellulose at velocities similar to those of wild-type yeast actin. Without methylcellulose, mutant filaments dissociated from the assay surface upon addition of ATP with little or no sliding detected. In contrast to this, filaments with alanines substituted at D80/D81 or E83/K84 were motile in the presence and absence of methylcellulose. Direct binding measurements involving cosedimentation of D24A/D25A and E99A/E100A actins with myosin subfragment-1 (S-1) in the presence of ATP revealed 3- and 2-fold decreases in their binding constants, respectively, compared to wild-type actin. In the absence of ATP all yeast actins had a similar affinity for S-1. A large decrease in the activation of S-1 ATPase was observed for both D24A/D25A and E99A/E100A actins. The D80A/D81A and E83A/K84A actin filaments showed normal S-1 binding and activation of ATPase activity. These results demonstrate the involvement of the D24/D25 and E99/E100 charged residues in the weak binding of myosin to actin and reveal that D80/D81 and E83/K84 residues in the 79−92 helix do not modulate actomyosin interactions.

Subdomain-1 of actin is thought to be the primary binding site for myosin during the cross-bridge cycle (Reisler, 1993; Rayment *et al.*, 1993). In order to better understand the role of this region in the mechanism of cross-bridge action, a complete description of changes at the actomyosin interface is required. The pursuit of this goal is complicated by the possibility that the interface involves multiple contacts between the two proteins. Recently, modeling of the S-1[1] and actin crystal structures in conjunction with electron microscopy reconstruction of S-1-decorated actin filaments has placed the charged residues of subdomain-1 at the actin−myosin interface (Rayment *et al.*, 1993; Shroder *et al.*, 1993). However, this fit was done for the strongly (rigor) bound acto-S-1 complex, while the charged residues are considered mainly responsible for the ionic strength dependent weak acto-S-1 binding in the presence of ATP. The primary electrostatic interaction is thought to involve six negatively charged residues on actin, at positions 1−4 and 24−25, and 5 lysine residues found at the 50/20-kDa junction on rabbit skeletal S-1 (Cheung & Reisler, 1992; Rayment *et al.*, 1993; Uyeda *et al.*, 1994). Modeling of S-1 into the actin filament also suggested a second interaction site, between the positively charged segment on S-1, 567−578, and the glutamic acid residues 99/100 on actin.

The first component of the actomyosin interface to be investigated was the extreme N-terminus of actin. Cross-linking, biochemical, and immunochemical approaches established the involvement of this area in interactions with myosin in the presence of ATP (Sutoh *et al.*, 1982; Bertrand *et al.*, 1989; DasGupta & Reisler, 1989, 1992). In addition, replacement of aspartic acid residues 1 and 4 with histidines diminished the $V_{max}$ of the actin-activated myosin ATPase without significantly changing the $K_m$ value (Sutoh *et al.*, 1991). Unambiguous evidence for the importance of the N-terminus on actin was provided by the enhancement of activation of S-1 ATPase through addition of two negatively charged residues to the yeast actin N-terminus (Cook *et al.*, 1993).

Techniques similar to those used in the studies on the N-terminus of actin have implicated other regions of subdomain-1 in functional interactions. Antibodies directed against the 18−29 region inhibited the S-1 binding to actin and the activation of S-1 ATPase (Adams *et al.*, 1993). Substitutions of aspartic acids at residues 24/25 in the 21−29 loop as well as glutamic acids at residues 99/100 in the 92−103 loop severely hampered the sliding of actin in the *in vitro* motility assays (Johara *et al.*, 1993). Also, synthetic peptides of sequence 77−95 were found to bind S-1 in the presence of ATP, implying the involvement of this region in the weak binding of S-1 to actin (Alessi *et al.*, 1992). However, the actual role of the charges in these regions, especially with respect to different steps of the cross-bridge cycle, remains undetermined.

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[1] Abbreviations: DNaseI, deoxyribonuclease I; F-actin, filamentous (polymerized) actin; G-actin, monomeric actin; HMM, heavy meromyosin; S-1, myosin subfragment-1.

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Wertman *et al.* (1992) investigated structure−function relationships of actin using cell growth as a biological assay. This work involved charged to alanine scanning mutagenesis of yeast *Saccharomyces cerevisae* actin in which surface charge pairs throughout the entire primary sequence were replaced with alanines. The mutant yeast strains were homozygous for the altered actins, allowing a direct assessment of their cellular properties. It is an advantage of the yeast system, which has a single actin gene, that results of *in vivo* and *in vitro* experiments can be combined to better understand the relationship of the structure and biological function of actin. Indeed, yeast strains with actins containing substitutions at the myosin binding site showed heat- and cold-sensitive growth. Further analysis of these mutants revealed deficiencies in cellular functions such as actin organization, cell division, and mitochondrial organization (Drubin *et al.*, 1993). Strikingly, a similar phenotype had been observed for *S. cerevisae* myosin mutants, giving additional evidence for the importance of the actomyosin system in these cellular processes (Johnston *et al.*, 1991).

In this study we took advantage of the conservative nature of alanine substitutions to show that the elimination of the D24/D25 and E99/E100 charged pairs decreases the weak binding of actin to myosin in the presence of ATP but does not abolish its motile function. However, this deficiency is sufficient to inhibit *in vitro* motility at conditions that allow filament diffusion from the myosin-coated surface. In contrast to this, the two charged pairs in the 79−92 helix, D80/D81 and E83/K84, do not modulate the functional interactions of actin with myosin.

## MATERIALS AND METHODS

*Reagents.* Distilled and Millipore-filtered water and analytical grade reagents were used in all experiments. ATP, PMSF, DTT, phalloidin, rhodamine phalloidin and $\beta$-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO). Yeast extract, dextrose, and bactopeptone were purchased from Difco (Detroit, MI). DNase I was purchased from Boerhinger Mannheim (Indianapolis, IN).

*Preparation of Proteins.* Yeast strains were a generous gift of Drs. D. Botstein, D. Drubin, and K. Wertman. The construction of these yeast strains was described by Wertman *et al.* (1992). Actin was isolated from each strain using DNase I affinity chromatography as previously described (Cook *et al.*, 1993). Rabbit actin and myosin were prepared from rabbit skeletal muscle according to the methods of Spudich and Watt (1971) and Godfrey and Harrington (1970), respectively. Myosin subfragment-1 was prepared according to Weeds and Pope (1977).

*Polymerization of Actins.* Polymerization of each of the actins by $MgCl_2$ was observed by measuring the light scattering at 325 nm in a Spex Flourolog spectrophotometer (Spex Industries Inc., Edison, NJ). The final light-scattering values for polymerization reactions were measured following incubation of samples for 24 h. A portion of each polymerized sample was removed and diluted under the polymerization conditions to observe by electron microscopy.

*Actin-Activated S-1 ATPase Assays.* Actin-activated ATPase activity was measured by using the malachite green assay (Kodama *et al.*, 1986). Because of the assay sensitivity, it was necessary to minimize free phosphate in the stock actin solutions (Sutoh *et al.*, 1991). Actins were polymerized in a solvent containing 4 mM $MgCl_2$, 5 mM KCl, 10 $\mu$M phalloidin, and 10 $\mu$M imidazole at pH 7.4 and centrifuged in a Beckman airfuge for 15 min. The resulting pellet was dispersed in the same buffer and the procedure was repeated. The resulting solution was used as F-actin. Actin samples at final concentrations from 2 to 30 $\mu$M were preincubated with 0.4 $\mu$M S-1 for 20 min in ATPase buffer (5 mM KCl, 2 mM $MgCl_2$, and 10 mM imidazole, pH 7.4. The ATPase reaction was initiated by addition of ATP to 1 mM final concentration. The reactions were stopped with an equal volume of 0.6 M perchloric acid and then diluted appropriately while maintaining a final concentration of 0.3 M perchloric acid. The mixture was centrifuged at 14 000 rpm for 10 min in a microcentrifuge to remove precipitated proteins. Amounts of released phosphate were quantified using the malachite green reagent (Kodama *et al.*, 1986), and the activity of S-1 alone was subtracted from each determined value. ATPase assays carried out in the presence of methylcellulose were done in similar fashion with addition of enough 2% methylcellulose stock to achieve a final concentration of 0.7%.

*In Vitro Actin Motility Assays.* The motility assays were performed as previously described (Cook *et al.*, 1993) with a few modifications. Temperature was maintained at 30 °C for all assays. HMM was prepared as described in Kron *et al.* (1991). In order to remove ATP-insensitive heads, HMM was centrifuged with 0.15 mg/mL phalloidin- stabilized actin in a solution containing 0.1 M KCl, 10 mM sodium phosphate, 5 mM MgCl, and 3 mM MgATP for 20 min in a Beckman Airfuge. The supernatant was applied to nitrocellulose-treated coverslips with an HMM concentration of 0.1 mg/mL. Rhodamine phalloidin has a tendency to dissociate off yeast actin filaments after dilution in assay buffer over time (Kron *et al.*, 1991). To circumvent this problem, a 3-fold molar excess of rhodamine phalloidin was used to maximize labeling. Rhodamine phalloidin-labeled actin filaments were added to the coated coverslips at 10 nM, and after 1 min the unbound filaments were washed away with the assay buffer (25 mM KCl, 1 mM EGTA, 4 mM $MgCl_2$, 10 mM dithiothreitol, and 10 mM imidazole, pH 7.4). Movement was initiated with the assay buffer containing 1 mM ATP and an oxygen-scavenging system. Samples contained methylcellulose at a final concentration of 0.7%. Methylcellulose was prepared by dissolving it in assay buffer and dialyzing for 48 h. Quantification of the sliding velocities was done with an Expertvision system (Motion Analysis, Santa Rosa, CA). The velocities of individual filaments with standard deviations of less then 1/3 of the average velocity were used for statistical analysis (Homsher *et al.*, 1992). Such filaments were considered to move smoothly in the assay system. No differences in percentage of filaments moving were seen in any of the samples. Copolymers were made from G-actin stocks, polymerized at 10 $\mu$M final concentration with 3 mM $MgCl_2$, and labeled as described above.

*Cosedimentation Assays.* Cosedimentation assays for binding of S-1 to 3 $\mu$M phalloidin-stabilized actin were carried out at 23 °C in 3 mM ATP, 3 mM $MgCl_2$, 25 mM KCl, and 10 mM imidazole, pH 7.4, as previously described (DasGupta & Reisler, 1992). When used, ATP was added after the incubation of proteins for 10 min. The protein samples were centrifuged in a Beckman Airfuge at 140 000*g* for 10 min. Similar assays were done for the strong binding

of S-1 to actin in 100 mM KCl and 10 mM imidazole, pH 7.4. Resuspended pellets and supernatants of each sample were examined on SDS−PAGE (Laemlli *et al.*, 1970). Pellets contained actin and S-1 bound to it; unbound S-1 was found in the supernatant. Under these conditions, S-1 was not pelleted when centrifuged in the absence of actin. Gels were stained with Coomassie Blue, and the bands were quantified by a Biomed Instruments Soft laser densitometer (Fullerton, CA) interfaced to an IBM-compatible 386 computer for integration of peak areas. The densitometric traces of pelleted protein bands were analyzed to determine the molar ratios of S-1 bound to actin. Molar stain ratios for S-1 and actin were obtained from appropriate calibration gels.

## RESULTS

*Purification and Polymerization of Mutant Actins.* Mutant strains were obtained from a bank of actin mutants created by the charged to alanine scanning mutagenesis method (Wertman *et al.*, 1992). Selected charged pairs throughout the entire actin sequence were replaced with alanines, and the gene was expressed in a haploid yeast strain. Four of these mutants were selected for *in vitro* study on the basis of their relationship to the myosin binding site on actin. Two of the mutation sites, D24/D25 and E99/E100, are in the middle of surface loops in subdomain-1, while the other two sites, D80/D81 and D83/K84, are in the 79−92 helix. Yeast strains that only expressed these mutant actins were both heat and cold sensitive. In addition, Drubin *et al.* (1993) found cell functions such as mitochondrial organization to be deficient for the D24/D25 and E99/E100 mutants.

The purification process for each actin strain yielded similar amounts of homogeneous protein, indicating no significant changes in the expression of actin or in the interaction with DNase I. Direct tests of that interaction on two of the mutants, D80A/D81A and E99A/E100A, showed inhibition of the DNase I activity equal to that by wild-type actin (data not shown). The ability of each mutant actin to polymerize was checked by monitoring the increase in light scattering after addition of $MgCl_2$ (Figure 1). The substitutions D80A/D81A and E83A/K84A had opposite effects on the rate of polymerization, with the former accelerating and the latter slowing the rate compared to that of wild-type actin. However, the extent of polymerization for both the D80A/D81A and E83A/K84A actins was similar to that of wild-type actin, indicating no significant change in critical concentrations of these mutants. Actins changed in the loop regions, D24A/D25A (Figure 1) and E99A/E100A (data not shown), were not affected in either the rate or extent of their polymerization.

The morphology of mutant filaments polymerized with $MgCl_2$ was inspected by electron microscopy (data not shown). All mutants showed normal filament structure except that the D24A/D25A filaments had some tendency to bundle. Bundling has been reported for actins mutated at both the N-terminus for yeast actin (Cook *et al.*, 1992) and residues E99/E100 for *Dictyostelium* actin (Johara *et al.*, 1993). Surprisingly, substitution of D24/D25 with histidines was not reported to induce bundling. After labeling with rhodamine phalloidin all actins exhibited normal filament appearance in the fluorescence microscope with occasional bundling detected for the D24A/D25A filaments. Clearly,

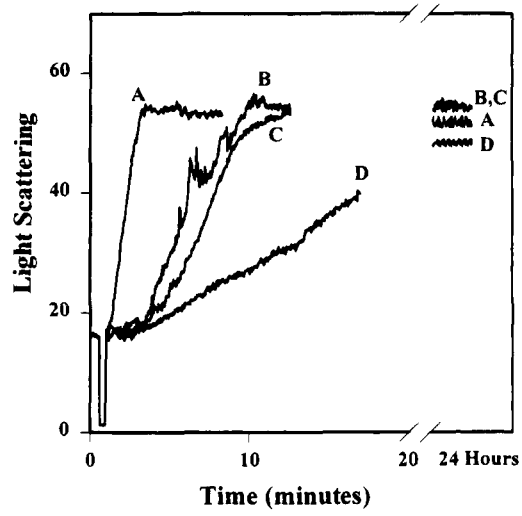


FIGURE 1: Polymerization of wild-type and mutant actins with $MgCl_2$. The polymerization of 3.0 $\mu$M mutant and wild-type G-actins in G-actin buffer (2 mM Tris, pH 7.6, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM $\beta$-mercaptoethanol) was detected by the increase in light scattering after addition of 3.0 mM $MgCl_2$. The initial polymerization was observed for 20 min for each actin. The final amount of polymerization was determined by measuring light scattering after incubation for 24 h. (A) D80A/D81A; (B) WT; (C) D24A/D25A; (D) E83A/K84A.

each of the mutants is competent to form filamentous actin, although the possibility of some differences in their substructure cannot be excluded.

*Sliding Velocities of Actin Filaments.* A previous study (Johara *et al.*, 1993) demonstrated that charge reversion of residues D24/D25 by substitution with histidines produced mutant actin filaments that were immotile. These filaments formed rigor bonds to the HMM-coated surface but dissociated without sliding after addition of ATP. In addition, similar replacement of glutamic acids at E99/E100 with histidines produced filaments that moved at velocities reduced to 17% of that of wild-type actin. The importance of D24/D25 and E99/E100 charges was reevaluated by using the alanine mutants of these residues. The advantages of alanines over histidines are related to the neutral charge and size of the former structure. In the *in vitro* motility assays D24A/D25A and E99A/E100A filaments remained bound to the HMM-coated surface after addition of ATP. Greater than 90% of D24A/D25A and E99A/E100A mutant filaments moved at velocities similar to wild-type ($3.8 \pm 0.2$, $4.0 \pm 0.2$, and $3.8 \pm 0.2$ $\mu$M/s, respectively; Figure 2, Table 1). In addition, the D80A/D81A and E83A/K84A filaments also moved at velocities similar to that of wild-type actin (Table 1). These results show that the eliminated charged residues are not essential for sliding of actin over myosin in the *in vitro* motility assay. However, there were noticeable differences in motility patterns of the D24A/D25A and E99A/E100A mutant filaments compared to wild-type actin. The tail portion of translocating mutant filaments failed to trace the path of the leading edge of the filament, a characteristic feature of wild-type filament motility. Instead, the back-end "waved" back and forth while the front portion was moving smoothly. Nevertheless, filaments undergoing this phenomenon moved at wild-type-like velocities. Such a motion was observed before for rabbit skeletal actin filaments moving over sparsely covered myosin surfaces and was attributed to a decreased binding of the two proteins (Uyeda *et al.*, 1990).

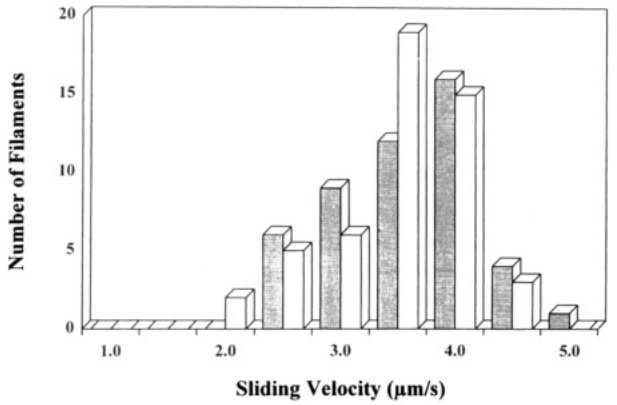


FIGURE 2: *In vitro* motilities of D24A/D25A mutant actin and wild-type actin in the presence of 0.7% methylcellulose. *In vitro* motilities of mutant (shaded bars) and wild-type actin (open bars) were measured over HMM in 10 mM imidazole, pH 7.4, 25 mM KCl, 1 mM EGTA, 4 mM $MgCl_2$, 10 mM dithiothreitol, and 0.7% methylcellulose on a nitrocellulose-coated surface. The mean velocities, standard deviations, and 95% confidence levels are given in Table 1.

Table 1: Sliding Velocities of Actin Filaments in the *in Vitro* Motility Assays[a]

| actin | MC | mean (μm/s) | SD | confidence level (95%) |
|---|---|---|---|---|
| WT | + | 3.8 | 0.9 | 0.2 |
| D80A/D81A | + | 3.9 | 0.7 | 0.2 |
| E83A/K84A | + | 3.7 | 0.6 | 0.2 |
| D24A/D25A | + | 3.8 | 0.6 | 0.2 |
| E99A/E100A | + | 4.0 | 0.7 | 0.2 |
| WT | − | 3.6 | 0.8 | 0.2 |
| D80A/D81A | − | 3.7 | 0.8 | 0.2 |
| D83A/D84A | − | 3.8 | 0.7 | 0.2 |
| D24A/D25A | − | no motile filaments observed | | 0.2 |
| E99A/E100A | − | no motile filaments observed | | 0.2 |

[a] The presence (+) and absence (−) of methylcellulose in the *in vitro* motility assays is indicated in the MC column. The motilities of actin filaments were measured as described in Materials and Methods. Fifty filaments were analyzed for each sample.

In order to test the affinity of these mutant actins for myosin, the motility experiments were repeated in the assay buffer lacking methylcellulose. Methylcellulose is a viscosity-enhancing agent included in the assay buffer to decrease dissociation of actins from myosin and enable myosin heads to cycle at their maximum rate (Uyeda *et al.*, 1990; Homsher *et al.*, 1992). In the absence of methylcellulose the wild-type filaments remained bound to the surface in the presence of ATP and moved with a distribution of velocities (3.6 ± 0.2 μm/s) similar to that in the presence of methylcellulose (Table 1). The same was true for the D80A/D81A and E83A/K84A mutant actin filaments, which remained bound in the absence of methylcellulose and moved with velocities (3.7± 0.2 and 3.8 ± 0.2 μm/s) similar to that of wild-type actin (Table 1). In contrast to this, without methylcellulose the D24A/D25A and E99A/E100A filaments were released from the surface of the coverslip after addition of ATP with little or no sliding detected (Table 1). This illustrates the importance of the charged pairs on the surface loops 21−29 and 92−103 of subdomain 1, but not in the 79−92 helix in the weak interactions with myosin.

To test whether the above charged pairs on actin contribute to weak actomyosin binding under conditions closer to a physiological environment, the *in vitro* motilities of wild-type and D24A/D25A actins were determined at different combinations of ionic strength and methylcellulose concentration. In the presence of 0.4% methylcellulose and at $\mu$ = 80 mM (adjusted to that value with KCl) about 50% of the wild-type actin filaments moved smoothly (see Materials and Methods) over the HMM surface with a mean velocity of 3.83 ± 0.74 μm/s. The remaining 50% of wild-type filaments detached from HMM for a considerable fraction of time, underwent Brownian motion, and displayed a stop-and-go type of motion. Under the same conditions only 20% of D24A/D25A actin mutant filaments moved smoothly ($v$ = 2.52 ± 1.14 μm/s, $n$ = 160), indicating that the charge pair D24/D25 contributes to the weak actomyosin binding also at intermediate ionic strength conditions ($\mu$ = 80 mM).

It has been suggested that only a limited number of actomyosin interactions along a filament are required to prevent its dissociation and translocate it over the myosin surface (Huxley, 1990). If this is true, and if the main damage in the D24A/D25A and E99A/E100A mutants is in their weak binding to myosin, then interspersing these mutants with wild-type actin in copolymer filaments should restore normal motility in the absence of methylcellulose. For filaments composed of 20% wild-type and 80% E99A/E100A or D24A/D25A a small fraction of filaments (less than 20%) remained bound to the surface in the presence of ATP and moved. However, upon increasing the proportion of wild-type actin to 50% the association of both the D24A/D25A and E99A/E100A actin copolymers improved dramatically, with nearly all of the filaments remaining bound and moving at sliding velocities similar to that of wild-type actin.

*Rigor- and Nucleotide-Sensitive Binding of the Mutant Actins to S-1.* The affinity of mutant actins for myosin under both strong and weak binding conditions was tested in cosedimentation experiments. The binding constants were obtained from the slopes of the linear binding plots shown in Figure 3. In the presence of MgATP, the binding constant of S-1 to D24A/D25A mutant (1.5 ± 0.2 × $10^4$ $M^{-1}$) was reduced by 3-fold compared to wild-type (4.1 ± 0.3 × $10^4$ $M^{-1}$), while that for the E99A/E100A mutant was decreased by 2-fold (2.2 ± 0.2 × $10^4$ $M^{-1}$) (Figure 3). Addition of 0.7% methylcellulose increased the affinity of S-1 for the D24A/D25A actin to a value (4.2 ± 0.3 × $10^4$ $M^{-1}$) very similar to that of wild-type actin in the absence of methylcellulose. The substitutions D80A/D81A and E83A/K84A did not have a significant effect on the binding of S-1 to actin, indicating that the loss of these charges did not affect interactions with myosin. Under rigor conditions, no effect on the binding of S-1 to actin was observed for any of the mutants used in this work (Figure 4).

*Actin-Activated ATPase Activities.* Replacement of aspartic acids with histidines at D24/D25 resulted in a complete loss of the activation of HMM ATPase activity by actin (Johara *et al.*, 1993). Replacement of these residues with alanines also reduced greatly the activation of S-1 ATPase, although some increase in the activity was detected with increasing actin concentrations (Figure 5). However, we were unable to approach the saturation of ATPase activation upon increasing the actin concentration to 30 μM. Thus, it was not possible to assess the effect of the D24A/D25A mutation on the $V_{max}$ and $K_m$ values of the acto-S-1 system, although clearly the $K_m$ must be greater than 30 μM. Activation of S-1 ATPase by the E99H/E100H actin was not determined before because of the bundling of this mutant

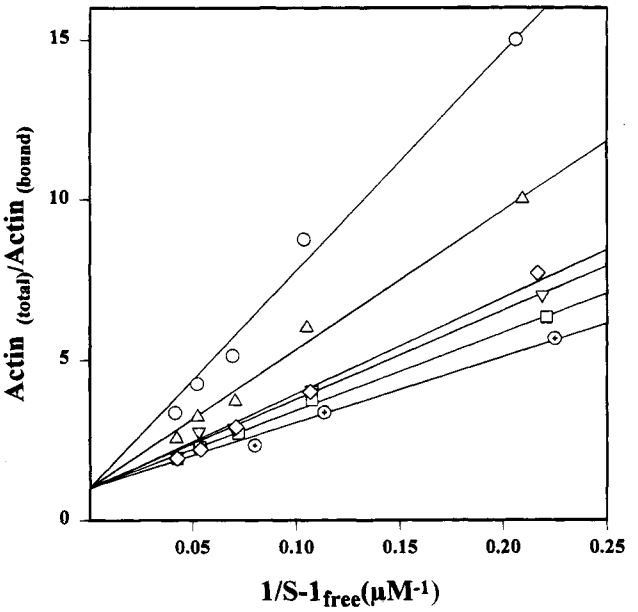


FIGURE 3: Cosedimentation of S-1 and wild-type and mutant actins in the presence of MgATP. Pelleting experiments were done with S-1 at concentrations between 3.0 and 20.0 $\mu$M and 3 $\mu$M wild-type and mutant actins. Proteins were pelleted in a solvent containing 10 mM Imidazole, pH 7.4, 25 mM KCl, 1 mM EDTA, 3.0 mM MgATP, and 3.0 $\mu$M phalloidin at 22 °C in a Beckman Airfuge at 140 000*g* for 10 min. Supernatants and pelleted samples were run on SDS−PAGE and quantified by densitometry. The following binding constants were determined for wild-type and mutant actins: wild-type actin (□), (4.1 ± 0.3) × $10^4$ $M^{-1}$; D80A/D81A(▽), (3.6 ± 0.2) × $10^4$ $M^{-1}$; E83A/K84A (◇), (3.4 ± 0.2) × $10^4$ $M^{-1}$; E99A/E100A (△), (2.2 ± 0.2) × $10^4$ $M^{-1}$; D24A/D25A (○), (1.5 ± 0.2) × $10^4$ $M^{-1}$; D24A/D25A with 0.7% MC (⊕), (4.2 ± 0.3) × $10^4$ $M^{-1}$.

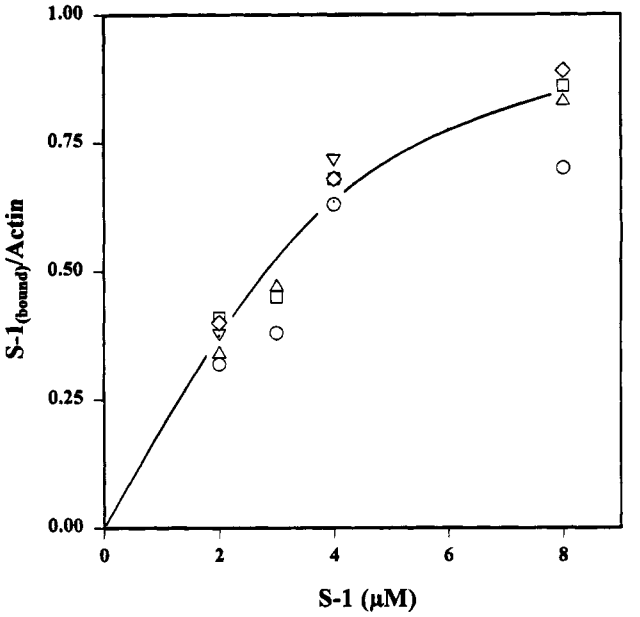


FIGURE 4: Cosedimentation of S-1 and wild-type and mutant actins under rigor conditions. Pelleting experiments were done with between 2.0 and 8.0 $\mu$M S-1 and 4.0 $\mu$M wild-type and mutant actins. The proteins were incubated in 100 mM KCl, 4.0 $\mu$M phalloidin, and 10 mM imidazole, pH 7.4 at 22 °C, and pelleted in a Beckman Airfuge. Supernatants and resuspended pellets were run on SDS−PAGE and quantified by densitometry. The symbols correspond to the following actins: wild-type actin (□), D80A/D81A (▽), E83A/K84A (◇), E99A/E100A (△), and D24A/D25A-(○). The solid line corresponds to a binding curve calculated for $K_a = 1.1 \times 10^6$ $M^{-1}$.

(Johara *et al.*, 1993). We measured a decrease in the activation of S-1 ATPase by the E99A/E100A actin similar to that of the D24A/D25A actin, with no indication of any saturation of ATPase activity up to 30 $\mu$M actin. As was true for other measurements of acto-S-1 interactions, the activation of S-1 ATPase by D80A/D81A and E83A/K84A actin filaments was the same as that for wild-type actin filaments. These data could be adequately described by $V_{max}$ = 3.0 ± 0.5 $s^{-1}$ and $K_m$ = 19.6 ± 6.3 $\mu$M values. Because of the dramatic effect of methylcellulose on the *in vitro* motility assay we also tested the activation of S-1 ATPase by the D24A/D25A actin with 0.7% methylcellulose included in the assay buffer. A slight increase in ATPase activation was observed for each concentration of actin tested, albeit not to wild-type actin levels.

## DISCUSSION

Prior to any functional evaluation of a mutant protein, it is important to address the possibility of structural deformation of the expressed protein outside the mutagenesis site. This is especially true for proteins displaying considerable structural flexibility and dynamic properties. Recent reports place actin in a category of such dynamic structures (Orlova & Egelman, 1993; Strzelecka-Golaszeweska *et al.*, 1993; Muhlrad *et al.*, 1994). In this work, the risk of significant changes on actin is reduced by the choice of surface residues for mutagenesis and the use of alanines for substitutions of charged residues. Fortunately also, two simple reactions, polymerization of actin and rigor binding to myosin, provide satisfactory tests for the general integrity of actin's structure.

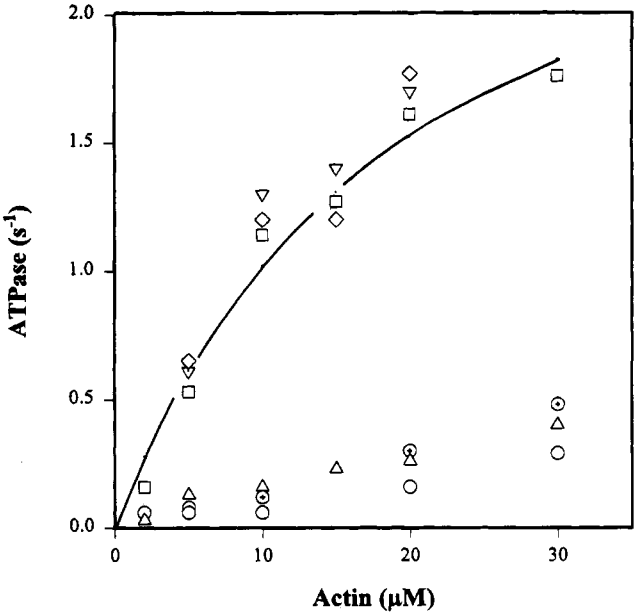


FIGURE 5: Actin-activated ATPase activities of S-1 in the presence of wild-type and mutant actins. The activation of S-1 ATPase by each actin was measured in the presence of between 2.0 and 30.0 $\mu$M phalloidin-stabilized F-actin, 0.4 $\mu$M S-1, and 1 mM ATP in an assay buffer containing 10 mM imidazole, pH 7.4, 5 mM KCl, and 2 mM $MgCl_2$. The symbols correspond to the following actins: wild-type actin (□), D80A/D81A (▽), E83A/K84A(◇), E99A/E100A(△), D24A/D25A(○), and D24A/D25A with 0.7% MC (⊕). The solid curve describes the fit of experimental data to the Michaelis−Menton equation ($V_{max}$ = 3.0 ± 0.5 $s^{-1}$; $K_m$ = 19.6 ± 6.3 $\mu$M).

The wild-type and each of the mutant actins formed similar amounts of morphologically similar filaments *in vitro*, although the rates of polymerization differed for the 79−93 helix mutants. Despite the proximity of the mutant sites D80A/D81A and E83A/K84A, the rates of their polymerization were changed in opposite directions relative to that of wild-type actin. The origin of this effect is unclear. However, since rates of actin polymerization are easily affected by many factors, the different kinetics do not imply that the filaments of these mutants are different.

Somewhat surprising was the appearance of occasional bundles for the D24A/D25A actin even though histidine substitution at the same site did not induce the bundling of actin filaments (Johara *et al.*, 1993). In contrast to that work, the E99A/E100A mutant filaments did not bundle, while the E99/E100 histidine mutants were reported to bundle. Besides these two charge pairs, bundling has also been induced by the reduction of charges at the N-terminus of yeast actin (Cook *et al.*, 1992). The inconsistency of the bundling of actin filaments resulting from different substitutions raises questions about the cause of this effect. Regardless of the bundling origin, it is clear that each of the yeast mutant actins can form normal filaments, and that the mutant effects *in vivo* cannot be attributed to the loss or impairment of filament formation. This conclusion is strengthened considerably by the observation that rigor binding of S-1 to wild-type and all mutant actins was very similar, if not the same. Thus, alterations in the sites examined in this work have little or no effect on the high-affinity binding of S-1 to actin.

The reversion of charges at residues D24/D25 and E99/E100 by replacement with histidines was shown to have a dramatic effect on the ability of actin to interact with myosin in the *in vitro* motility assay. Under the conditions of our motility assays, which minimize the dissociation of filaments off the surface, alanine mutants D24A/D25A and E99A/E100A were able to move at velocities similar to that of wild-type actin. However, in assay buffer lacking the viscous agent methylcellulose these two mutants dissociated off the assay surface upon addition of ATP. The motility of the histidine mutants was not measured in the presence of methylcellulose, leaving open the question whether these mutants would function at conditions minimizing acto-HMM dissociation (Johara *et al.*, 1993). Nonetheless, it is apparent that removal of the D24/D25 and E99/E100 charges is sufficient under some conditions to affect the motility of actin, emphasizing the importance of these charges in weak actomyosin interactions. Since the N-terminus of wild-type yeast actin is missing two of the four acidic charges of α-skeletal actin, the D24A/D25A and E99A/E100A mutants are functioning with four fewer charges than the rabbit actin. Yet, their movement in the presence of methylcellulose is equally fast, indicating that the D24/D25 and E99/E100 charged pairs, while important for the weak binding, are not critical for the steps subsequent to such binding in the transduction of ATP energy into motion. *In vitro* motility assays carried out under higher ionic strength conditions ($\mu$ = 80 mM) revealed that the D24/D25 charges contribute to weak actomyosin binding also at higher salt concentrations. This increases the probability that such interactions are physiologically significant. An interesting aspect of the motility of the E99A/E100A actin in the presence of methylcellulose is related to the recent modeling of the acto-S-1 complex in which the S-1 binds to two actin promoters in the filament (Milligan *et al.*, 1990; Rayment *et al.*, 1993). According to these studies, the binding site for S-1 on the "second actin" is centered around the 92−103 loop. Thus, it appears that a loss, at least partial, of this interaction does not impair the motile function of actin.

Previous work with a synthetic peptide of residues 77−95 implicated this region in weak interactions with S-1 (Alessi *et al.*, 1991). Our results suggest that elimination of charges in the 79−92 helix does not impair the motility, even under conditions at which the other mutants dissociate from HMM. Also, no effect on other aspects of interaction with myosin, such as its binding and activation of ATPase, was observed. This suggests that the mutant phenotypes for D80A/D81A and E83A/K84A do not result from decreased interactions of actin with myosin. However, a possible role in actomyosin interactions of other uncharged residues in the 79−92 helix cannot be excluded.

In agreement with the effects observed in the motility assays, cosedimentation experiments revealed a reduction in the binding constant of S-1 (+MgATP) to the D24A/D25A and E99A/E100A actins relative to its binding to wild-type actin. Moreover, the effect of methylcellulose on solution interactions appeared to correlate with the motility assay results as well. The binding constant of S-1 to the mutant actins was increased to a value similar to that of wild-type actin without methylcellulose. A complete restoration of wild-type-like motilities in 1:1 but not 1:4 mixtures of wild-type and mutant actins in the absence of methylcellulose can be rationalized also in terms of actin affinities for HMM. It appears likely that wild-type actin facilitates the interaction of mutant actins with HMM in mixed filaments by slowing the diffusion of the copolymer from HMM. The fact that in 1:4 mixtures of wild-type and mutant actins less than 20% of actin filaments are motile in the absence of methylcellulose may be related to the step-like dependence of actin movement on the surface density of HMM and the probability of acto-HMM binding. An example of such a dependence is the observation that upon increasing the ionic strength of the medium a critical point is reached at which filaments dissociate from the HMM surface (Homsher *et al.*, 1992).

Previous work showed that antibodies to the 18−29 region decreased weak myosin binding to actin and reduced the activation of the myosin ATPase (Adams & Reisler, 1993). In addition, the D24H/D25H *Dictyostelium* actin mutants did not activate the myosin ATPase (Johara *et al.*, 1993). Some, albeit low, activation of myosin ATPase was detected with D24A/D25A actin filaments. It is apparent from Figure 5 that the $K_m$ is increased for the D24A/D25A and E99A/E100A mutants, although the exact $K_m$ and $V_{max}$ values could not be determined from these results. An increase in the $K_m$ value for these mutants was also indicated by their decreased binding to S-1·ATP (Figure 3). However, changes in $K_m$ alone cannot account for the low acto-S-1 ATPase activities of the D24A/D25A and E99A/E100A mutants. A likely decrease in the acto-S-1 $V_{max}$ of the D24A/D25A mutant is suggested by the fact that addition of methylcellulose restores the weak binding but not the ATPase activity to wild-type actin levels. Unfortunately, the results of *in vitro* motility experiments cannot be mobilized in favor or against such a conclusion. This difficulty arises from the observation that differences in $V_{max}$ values as great as 5-fold do not necessarily change the velocity of actin filament motion (Cook *et al.*, 1993). Future experiments that

maximize the interaction of the two proteins may provide direct answers to the question of the catalytic role of the D24/D25 and E99/E100 charges.

Finally, a comparison of the effect of mutations in the 21−29 and 92−103 loops and at the N-terminus of actin on actomyosin interactions merits attention. In the latter case, substitution of acidic residues with histidines (Sutoh *et al.*, 1991) or addition of acidic residues to the wild-type yeast actin (Cook *et al.*, 1993) produces large changes in $V_{max}$ but not in $K_m$ values of the acto-S-1 ATPase. As shown by these authors the $K_d$ values of actin and S1•ATP are also unchanged by such manipulation of actin. This is indicative of the uncoupling between the weak myosin binding and myosin ATPase activating functions of actin as also suggested by the work with antibodies to the N-terminus of actin (DasGupta & Reisler, 1992). The implication of these results is that mutations or the blocking of the N-terminus by $F_{ab}$ inhibits a step subsequent to the weak actomyosin binding, probably the transition from weakly to strongly bound actomyosin complexes. Such a possibility is consistent with the complete inhibition of active force in muscle fibers incubated with the N-terminal actin antibodies and only a partial loss of their relaxed stiffness (Brenner *et al.*, 1993). On the other hand, as shown in this work, D24/D25 and E99/E100 charged pairs are important for the weak acto-S-1 binding, while their contribution to the catalytic function is not established yet.

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