Tryptophan Fluorescence of Yeast Actin Resolved via Conserved Mutations

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ABSTRACT Actin contains four tryptophan residues, W79, W86, W340, and W356, all located in subdomain 1 of the protein. Replacement of each of these residues with either tyrosine (W79Y and W356Y) or phenylalanine (W86F and W340F) generated viable proteins in the yeast *Saccharomyces cerevisiae*, which, when purified, allowed the analysis of the contribution of these residues to the overall tryptophan fluorescence of actin. The sum of the relative contributions of these tryptophans was found to account for the intrinsic fluorescence of wild-type actin, indicating that energy transfer between the tryptophans is not the main determinant of their quantum yield, and that these mutations induce little conformational change to the protein. This was borne out by virtually identical polymerization rates and similar myosin interactions of each of the mutants and the wild-type actin. In addition, these mutants allowed the dissection of the microenvironment of each tryptophan as actin undergoes conformational changes upon metal cation exchange and polymerization. Based on the relative tryptophan contributions determined from single mutants, a triple mutant of yeast actin (W79) was generated that showed small intrinsic fluorescence and should be useful for studies of actin interactions with actin-binding proteins.

INTRODUCTION

Actin, a key protein in many cellular processes in eukaryotic organisms, has been the subject of intensive investigations. It plays a pivotal role in cellular locomotion and in the cell cytoskeleton. In higher organisms, actin, along with myosin and other proteins, is necessary for muscle contraction, during which it interacts in a cyclic fashion with myosin heads (Cooke, 1997).

Many biochemical investigations into conformational changes on actin or myosin related to these processes have involved the labeling of the actin molecule with fluorescent probes, or other reporter groups (Feng et al., 1997; Kim et al., 1996, 1995; Miki et al., 1992; Sheterline et al., 1995). However, introduction of such groups to the surface of actin may affect its affinity for other proteins and substrates, and alter thereby its overall function. One example of such chemical modification "penalty" is the opposing effects of different probes, attached to C374 on actin, on actomyosin ATPase activity and the in vitro motility of actin filaments (Crosbie et al., 1994).

In many cases, the tryptophan fluorescence of proteins offers an attractive alternative to the use of extrinsic probes for monitoring local structural changes around the labeled residues (Burstein et al., 1973; Eftink and Ghiron, 1976, 1977). In actin, its intrinsic fluorescence has previously been used to monitor changes associated with metal cation exchange and actin polymerization (Selden et al., 1994). Such conformational changes result in an increase and decrease in tryptophan fluorescence, respectively, which in principle could be used to map these changes to specific sites on actin. However, this task is complicated by the fact that actin has four tryptophan residues, all of them located within subdomain 1 of the protein (see Fig. 1; Kabsch et al., 1990; Lorenz et al., 1993). Each of these residues is buried in the structure, separated through the depth of the protein, as viewed in the Lorenz et al. (1993) model for the actin protomer within filamentous (polymerized) actin (F-actin; compare Fig. 1 *a* and 1 *b*). The relative contribution of each tryptophan to the overall emission spectra is not apparent from the structure, although a prediction as to the quenching effects of neighboring sulfur atoms from cysteine and methionine residues has been advanced (Kuznetsova et al., 1996, 1999). In addition, fluorescence from any given tryptophan may be affected by other factors in the environment of the residue. For example, it has been proposed that fluorescence quenching of a tryptophan residue in the immunosuppressant drug FK506-binding protein (FKBP) is due to an atypical H-bond interaction between the indole nitrogen and a benzene ring from an adjacent phenylalanine residue (Rouviere et al., 1997). Similarly, fluorescent energy transfer between tryptophan residues, via a histidine residue, has been observed (Loewenthal et al., 1991). Thus, to describe the contribution of each of the tryptophans of actin to its overall fluorescence and map the conformational changes within the protein to specific tryptophans, conservative mutations should be targeted at each of the tryptophan residues.

Several other proteins have been subjected to such an analysis, e.g., glutamine synthetase (Atkins et al., 1991), barnase (Loewenthal et al., 1991), L-lactate dehydrogenase (Smith et al., 1991), acyl-ACP thioesterase (Li et al., 1998),

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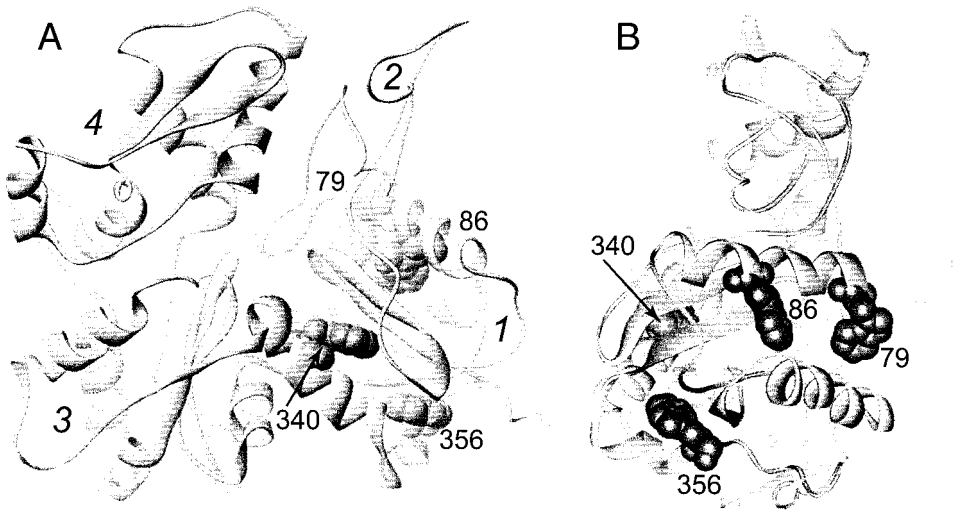

FIGURE 1 Location of the four tryptophan residues, 79, 86, 340, and 356, in subdomain 1 of the actin monomer. Tryptophan residues (shown in dark gray space filling) are shown from the front (*A*) and right hand (*B*) faces of the actin protomer (Lorenz et al., 1993). The right hand side view (*B*) shows the distribution of the tryptophan residues through the depth of subdomain 1. Subdomains are indicated by larger italicized numbers. Structure is displayed using WebLab ViewerLite, version 3.1 (MSI, San Diego, CA), on a Macintosh computer (Apple Computer Inc., Cupertino, CA).

phosphoglycerate kinase (Szpikowska et al., 1994), carbonic anhydrase II (Mårtensson et al., 1995), and myosin subfragment-1 (S1; Yengo et al., 1999, 1998). In these cases, conservative mutations of single and multiple tryptophans have generated functional proteins that allow accurate assignment of relative fluorescence contribution for each tryptophan residue. In addition, Smith et al. (1991) further mutated many tyrosine residues of the tryptophan-null (trp-null) L-lactate dehydrogenase to tryptophan, and the fluorescence characteristics of these mutants were described.

The purpose of this study has been to determine the contributions of individual tryptophan residues to actin fluorescence and to assign fluorescence changes observed upon divalent metal ion exchange and actin polymerization to specific tryptophan residues. Single tryptophan residues were conservatively mutated in yeast actin, and the fluorescence of each of the mutants was measured for different states of actin. Based on the results of these single mutants, a triple mutant was then constructed that had a single tryptophan, W79, which had been identified as having the smallest contribution to overall actin fluorescence. The small tryptophan fluorescence and myosin binding of this actin offer special advantages for probing local, actin-induced changes in tryptophan mutants of S1 (Yengo et al., 1999, 1998).

MATERIALS AND METHODS

Actin mutagenesis

The yeast actin gene was mutated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) in two plasmids, *pRB1456* (Wertman et al., 1992) and *pTD24* (Miller et al., 1996a). The former plasmid has an *Eco*RI genomic fragment containing the yeast *ACT1* gene, with the *HIS3* gene cloned into the *Bcl*I site 3′ to the actin coding region, cloned into the bacterial plasmid *pUC119*. *pTD24* contains the same *ACT1*/*HIS3* fragment subcloned into the yeast centromeric plasmid *pRS314* (Sikorski and Hieter, 1989). Mutagenesis was performed with oligonucleotide pairs W79Yf and W79Yr (GTCACCACCtatGACGATATG, CATATCGTCataGGTGGTGAC), W86Ff and W86Fr (GAAAAGATatttCATCATACC, GGTATGATGaaatATCTTTTC), W340Ff and W340Fr (GAAAGTAtTCCGTCtttATTGGTGGT, ACCACCAATaaaGACGGAATaCTTTC), and W356Yf and W356Yr (CCAACAAATGtatATCTCAAAAC, GTTTTGAGATataCATTTGTTGG). (Lowercase letters indicate mutated nucleotides.) In each case, mutagenesis removes a restriction site from the coding region (W79Y: Δ*Bsr*I, W86F: Δ*Bgl*II, W340F: Δ*Sca*I, and W356Y: Δ*Bst*YI) to enable rapid screening and identification of mutated plasmids. Conditions used for mutagenesis varied slightly from those in the protocol enclosed with the kit to compensate for the large size of plasmids used in this study: the amount of template plasmid DNA was increased to 100 ng/reaction, thermocycler cycling conditions were changed to 95°C for 10 s, 37°C for 1 min, 68°C for 20 min, and 25 cycles were performed during the mutagenesis reaction. The mutagenesis mixture was digested with *Dpn*I for 60 min at 37°C and transformed into *Escherichia coli*. Transformants showing restriction digests corresponding to mutated residues were confirmed by dideoxy-sequencing (GeneMed Inc, San Francisco, CA). Multiple tryptophan mutations were made by repeating the above mutagenic strategy with appropriate oligonucleotide pairs. Final mutants were confirmed by sequencing.

Yeast transformation and genetic manipulation

Yeast media and general manipulations were performed as described previously (Sherman, 1991). Yeast strain DBY5532 (MAT a/α, *TUB2-ACT1*/*tub2–201-act1::LEU2*, *leu2–3, 112*/*leu2–3, 112*, *his3Δ200*/*his3Δ200*, *ade2–201*/*ADE2*, *ade4*/*ADE4*, *ura3–52*/*ura3–52*, *CAN1*^{S}/*can1*^{R}) (Wertman et al., 1992) was transformed either with the complete mutated plasmid (*pTD*24) or the *Eco*RI fragment bearing the actin gene from the mutated *pTD*24 and *pRB*1456 plasmids using the method of Ito et al. (1983). The resulting strains were selected for histidine independence. Transformants were sporulated and haploid spores isolated by dissection (Sherman and Hicks, 1991). Strains bearing the mutant actin as the sole actin gene were selected by autotrophic marker scoring, and confirmed by colony polymerase chain reaction and restriction analysis of the amplified actin gene. Strains bearing the mutated actin gene on plasmids were used for this study (see Table 1).

TABLE 1 Actin strains and plasmids

Plasmids	
pTD24	Wild-type actin linked to HIS3, cloned into pTD314 (a derivative of pRS314)
pRB1456	Wild-type actin linked to HIS3, cloned into pUC119
pER1	pTD24 with W79Y mutation
pER3	pTD24 with W86F mutation
pER7	pTD24 with W340F mutation
pER11	pTD24 with W356Y mutation
pER49	pTD24 with W340F/W356Y mutations
pER76	pTD24 with W86F/W340F/W356Y mutations ("W79")
pER101	pTD24 with W79Y/W86F/W340F/W356Y mutations ("trp-null")
Yeast strains	
DBY5532	Yeast diploid strain with genomic, heterozygous deletion of the ACT1 gene
TDS480	W79Y expressing yeast strain; haploid segregant of DBY5532 with pER1
TDS481	W86F expressing yeast strain; haploid segregant of DBY5532 with pER3
TDS482	W340F expressing yeast strain; haploid segregant of DBY5532 with pER7
TDS483	W356Y expressing yeast strain; haploid segregant of DBY5532 with pER11
TDS711	W340F/W356Y actin expressed in haploid segregant of DBY5532 (pER49)
TDS723	W79 actin expressed in haploid segregant of DBY5532 (pER76)
TDS701	Trp-null actin expressed in haploid segregant of DBY5532 (pER101)

Protein purification

Yeast actin was purified from each strain by affinity chromatography on deoxyribonuclease I (DNase I) columns as described previously (Cook et al., 1993), except that 5.0 mM HEPES/NaOH, pH 7.6, 0.2 mM $CaCl_2$, 0.2 mM ATP, 2.0 mM β-mercaptoethanol was used as the G-buffer. Rabbit α-actin and myosin were isolated from skeletal muscle by the methods of Spudich and Watt (1971) and Godfrey and Harrington (1970), respectively. Heavy meromyosin and S1 preparations from myosin were according to the methods of Kron et al. (1991) and Weeds and Pope (1977), respectively. All proteins were used within 2 weeks of preparation.

Actin polymerization

Wild-type and mutant actins were polymerized by the addition of magnesium chloride to 4.0 mM to solutions of 3.0 μM actin. Polymerization was monitored by light scattering at 350 nm using a Spex Fluorolog double monochromator spectrofluorometer (Jobin Yvon-Spex Instruments S.A. Inc., Edison, NJ).

In vitro motility assays

Motility assays were performed as described before (Miller et al., 1996b). Actin filaments (3.0 μM) were labeled with rhodamine phalloidin (Molecular Probes, Eugene, OR) overnight. Filaments (20 nM) were applied to coverslips coated with heavy meromyosin (0.3 mg/ml) at 25°C and allowed to bind for 30 s. Unbound filaments were washed away with assay buffer (25 mM KCl, 1.0 mM EGTA, 4.0 mM $MgCl_2$, 10 mM DTT, 10 mM imidazole, pH 7.4). Movement was initiated by the addition of assay buffer containing 1.0 mM ATP and was recorded in the presence (0.5%, w/v) and absence of methylcellulose. Quantification of the sliding speeds was performed using an ExpertVision System (Motion Analysis, Santa Rosa, CA). Individual filaments sliding at speeds with standard deviations of less than half the average speed were considered to move smoothly in the assay system and were used for statistical analysis of motion.

Rigor binding of actin to S1

Cosedimentation assays of rigor binding of S1 to 4.0 μM phalloidin-stabilized actin were carried out at room temperature in 4.0 mM $MgCl_2$, 10 mM NaCl, 10 mM imidazole, pH 7.0, 5.0 mM β-mercaptoethanol, as previously described (Miller and Reisler, 1995). The concentrations of S1 ranged between 1.0 and 15 μM. The protein samples were centrifuged at room temperature in a Beckman airfuge at 140,000 rpm for 15 min. Resuspended pellets and supernatants from each sample were examined by sodium dodecyl sulfate-ployacrylamide gel electrophoresis (Laemmli, 1970). Gels were stained with Coomassie Blue and then scanned and quantified using SigmaGel (Jandel Scientific, Chicago, IL). The molar ratios of S1 bound to actin were obtained from these data and the appropriate calibration gels of protein stain. To yield the dissociation constant, K_d, of S1 from actin, the binding data were fitted to Eq. 1, which describes the binding of these proteins at 1:1 molar ratio:

$$S1_b = \frac{(S1 + A + K_d) - \sqrt{(S1 + A + K_d)^2 - 4A.S1}}{2} \quad (1)$$

where $S1$ and A are the total concentrations of S1 and actin, respectively, and $S1_b$ is the concentration of S1 bound to actin.

Tryptophan fluorescence scanning

Tryptophan emission spectra were recorded in the Spex Fluorolog spectrofluorometer at room temperature (22°C) between 300 and 450 nm, with the excitation monochrometer set at 295 nm. Rabbit, yeast wild-type, and mutant actins were first diluted to 4.0 μM in 5.0 mM HEPES/NaOH, pH 7.6, 20 μM $CaCl_2$ (final), 200 μM ATP, and 2.0 mM β-mercaptoethanol (Selden et al., 1994). Magnesium cation exchange was performed by the addition of EGTA (pH 8.0) and $MgCl_2$ to a final concentration of 100 μM each to the above solution of Ca-globular (monomeric) actin (G-actin). After a 6-min incubation, the tryptophan emission spectra of such solutions were recorded. Polymerization of Mg-G-actin was induced by further addition of $MgCl_2$ (1.0 μl) to 4.0 mM final concentration. Scans were performed in duplicate and averaged.

RESULTS AND DISCUSSION

Mutagenesis and yeast strain characterization

Four novel mutations in the yeast actin coding sequence were performed to substitute each of the four tryptophan residues (79, 86, 340, and 356, see Fig. 1) with either tyrosine (residues 79 and 356) or phenylalanine (residues 86 and 340). Phenylalanine and tyrosine were chosen because they have similar hydrophobic properties and bulk to tryptophan residues; hence, it was anticipated that such substitutions would confer minimal conformational changes on actin.

Oligonucleotides used to perform site-directed mutagenesis were designed to delete a restriction site within the actin coding region. In two cases, this was achieved simply by the substitution of the codon for tryptophan to tyrosine (TGG → TAT, W79Y, and W356Y). However, the other

two mutations (W86F and W340F) required a silent mutation at a second codon, which alters the DNA sequence, but not the amino acid encoded. These restriction site alterations allowed for the rapid screening of both *E. coli* and yeast transformants to determine colonies with mutant actins. Yeast expressing each of the single tryptophan mutant actins as a sole source grew as vigorously as wild-type yeast (data not shown).

On the basis of tryptophan fluorescence results of single mutants discussed below, double (W340F/W356Y), triple (W86F/W340F/W356Y, or simply W79), and quadruple (W79Y/W86F/W340F/W356Y, or trp-null) mutants were made. Each of these mutants conferred viable protein when expressed as the sole actin in yeast, although strains expressing W79 or trp-null actins showed slower growth than wild-type isogenic strains (data not shown).

Characterization of actin mutants

Wild-type and single tryptophan mutant actins were purified from yeast strains with similar yields (2.5 ± 0.5 mg actin/100 g cell mass). The triple mutant W79 actin yielded about half the actin compared with similar weights of wild-type yeasts. The trp-null actin did not yield any protein from three separate preparations and was not further investigated. To determine whether the single and triple tryptophan mutations confer any structural changes on actin, several assays were performed to test their biochemical properties. These included the measurements of polymerization, S1 binding, and the sliding of actin in the in vitro motility assays.

Polymerization of actin was initiated by the addition of magnesium chloride (4.0 mM) and was followed by light scattering at 350 nm. All mutant actins showed almost identical polymerization profiles to the wild-type yeast protein (data not shown). The substitution of tryptophan residues, therefore, does not affect actin self-assembly into filaments. This suggests that the above mutations do not perturb actin-actin interactions and, thus, most likely do not significantly alter the actin structure.

The binding of each of the actins to myosin in the absence of ATP (strong, rigor binding) was tested in cosedimentation assays. A slight difference between two of the single mutants and the other single mutants and wild-type actins was observed (Fig. 2). The data for the wild-type, W79Y, and W86F actins can be fitted to a single binding curve corresponding to a dissociation constant of 0.18 ± 0.06 μM, whereas the binding curve describing the W340F and W356Y actin data corresponds to K_d = 0.8 ± 0.4 μM. Within the accuracy of the data, this difference indicates a small, approximately fourfold, decrease in the affinity of the W340F and W356Y mutants to S1, compared with the other actins. Previous mutation of a hydrophobic residue adjacent to W340, namely I341A, was shown to cause a ninefold decrease in the binding between the two proteins (Miller et al., 1996a). This is consistent with molecular modeling of

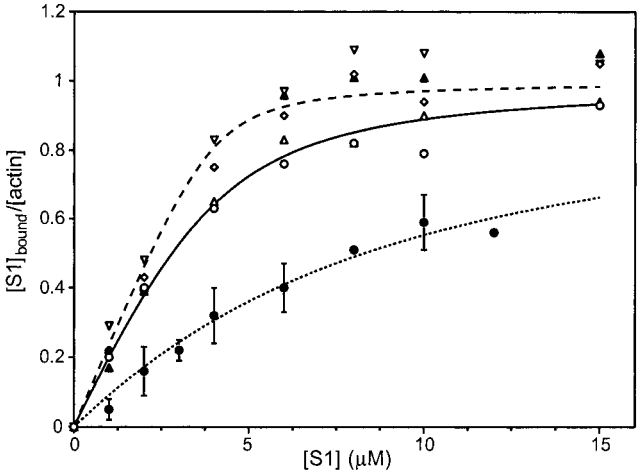

FIGURE 2 Rigor binding of S1 to wild-type and mutant actins (in the absence of nucleotides). The data for S1 binding to W340F (○) and W356Y (△) mutant actins are described by a single binding curve (*solid line*) with a dissociation constant of 0.8 ± 0.4 μM. S1 binding to wild-type (▲), W79Y (▽) and W86F (◇) actins is described by a single but separate curve (*dashed line*) with K_d = 0.18 ± 0.06 μM. S1 binding to W79 (●) actin is described by a third curve (*dotted line*) with K_d = 6.3 ± 0.4 μM. Error bars are omitted from all data except W79 actin for clarity.

the actomyosin complex which identified I341 as an amino acid involved in the strong binding of these proteins (Milligan, 1996; Rayment et al., 1993; Schröder et al., 1993). The observation that conservative substitutions of either W340 and W356 also decrease, albeit less, the rigor-S1 affinity suggests that these residues may also be involved in such a binding. The triple mutant W79, which has both W340 and W356 substitutions, shows a larger decrease in the strong binding affinity, with the binding data fitting a curve with a K_d = 6.3 ± 0.4 μM (Fig. 2). That the affinity decrease of W79 actin for S1 is somewhat greater (about 30-fold) than that expected from the product of changes caused by single W340F and W356Y mutations (about 20-fold) is not surprising. The effects of multiple site mutations on ΔG (and K_d) of protein-protein binding interactions are seldom strictly cumulative, reflecting either synergistic or antagonistic changes at the binding surface. Although the decrease in the affinity of W79 mutant actin for S1 is undesirable, this appears to be a minor disadvantage compared to the spectroscopic advantages of using this actin in acto-S1 studies.

In order to ascertain the effects of the single tryptophan mutations on the overall cycle of actomyosin interactions, in vitro motility assays were performed in the presence and absence of the viscosity-enhancing agent methylcellulose. The speeds of the actin filaments in these assays in the presence of methylcellulose were virtually identical (Table 2), showing similar functional properties of wild-type and mutant actins. Filament speeds were also essentially unaltered by tryptophan mutations on actin in the absence of methylcellulose (data not shown). The deletion of methyl-

TABLE 2 Sliding speeds of yeast actin in the in vitro motility assays

Actin	Filament speed (μm/s)
Wild-type	2.89 ± 1.10 (n = 115)
W79Y	3.00 ± 0.95 (n = 196)
W86F	3.18 ± 0.96 (n = 218)
W340F	2.96 ± 0.95 (n = 73)
W356Y	2.98 ± 0.97 (n = 147)

Mean speeds (± standard deviations of speed) of actin filaments moving on HMM-covered cover slips in the in vitro motility assays. The filament speeds data are shown in the presence of 0.5% (w/v) methylcellulose, with number of filaments analyzed indicated in parentheses. Similar speeds were recorded for all actins in the absence of methylcellulose.

cellulose from the motility assays can be used to identify actin weak binding mutants (Miller and Reisler, 1995). In the absence of this viscosity-enhancing agent, the actin filaments diffuse away from the heavy meromysosin in the case of weak binding defects. All single tryptophan mutant actins used in this study were unaffected by methylcellulose, confirming that there is no significant change in the weak binding of the mutants compared to wild-type actin. Filaments of W79 actin also moved well in the in vitro motility assays (data not shown). Thus, the near identical sliding speeds of the mutant and wild-type actins under both conditions show that the mutations do not affect the overall cross-bridge cycle and suggest that the actin function is similar for all of the actins tested.

The results of actin polymerization experiments, S1 binding, and in vitro motility assays suggest that neither the structure nor the function of the single tryptophan mutants examined in this study are significantly different from those of wild-type actin. Therefore, the mutants used in this work, and perhaps other mutants with multiple tryptophan substitutions, may represent useful tools to investigate conformational transitions in actin and actomyosin interactions. In addition, it is apparent that although the gross physiology of the actin molecule is not altered by the tryptophan mutants, some changes in myosin binding occur in the W340F, W356Y, or W79 mutants.

Fluorescence properties of tryptophan mutant actins

Previous studies have identified the intrinsic tryptophan fluorescence of actin as a convenient tool to monitor conformational changes in the molecule as it undergoes metal ion exchange and polymerization (Selden et al., 1994). Specifically, substitution of calcium with magnesium ions in the nucleotide cleft on actin induces a fluorescence increase, whereas the polymerization of actin by increased magnesium concentration decreases tryptophan fluorescence. The single tryptophan mutants described above provide experimental material for resolving the contributions of each of the four tryptophans on actin to its overall fluorescence and for determining their role in fluorescence changes associated with structural transitions in actin.

A comparison of the fluorescence emission scans of wild-type and the four single tryptophan mutant actins provides an indication of the contribution of each tryptophan residue to the intrinsic fluorescence of actin. Such a comparison, which is shown in Fig. 3 for Ca^{2+}-G-actin, reveals that each tryptophan makes a different contribution to the intrinsic fluorescence of the wild-type protein.

Table 3 summarizes both the results shown in Fig. 3 (not normalized) and those of six different experiments averaged and normalized to 100%. According to these results, both tryptophans 79 and 86 show little contribution (1% and 11%, respectively) to the overall fluorescence of actin. This had been predicted on the basis of the proximity of W79 and W86 to cysteine and methionine residues M82, -115, -119, and C10 (C17 in yeast) (Kuznetsova et al., 1996, 1999). It was proposed that the sulfur atoms would quench the fluorescence of these tryptophan residues, which appears to be the case. Similarly, Kuznetsova et al. (1996, 1999) predicted that the buried tryptophans 340 and 356 would dominate the fluorescence spectra of actin. In our experiments, these two tryptophans contribute similarly to the intrinsic fluorescence of actin (37% and 51%, respectively).

It is notable, also, that the substitution of any of the four tryptophans does not change the λ_{max} value of the emission spectra (λ_{max} = 328 nm). Kuznetsova et al. (1996) suggested that the relative degree of solvent exposure of residues 79 and 86 compared to 340 and 356 would cause the

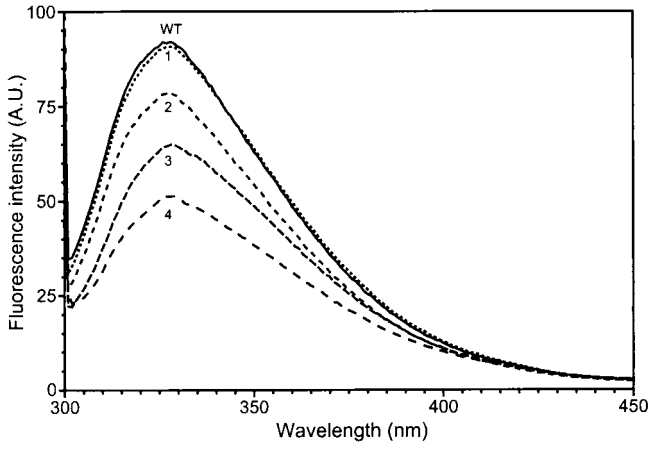

FIGURE 3 Fluorescence emission scans of wild-type and mutant actins in the Ca^{2+}-G-actin form. Actins were diluted to 4.0 μM in 5.0 mM HEPES/NaOH, pH 7.5; 200 μM ATP, 20 μM $CaCl_2$, 2.0 mM mercaptoethanol, and emission spectra (300–450 nm) were obtained with the excitation wavelength set at 295 nm. *Solid line* shows the spectrum of the wild-type actin (WT, line 1), and *dashed lines* represent the spectra of the four mutant proteins (1, W79Y; 2, W86F; 3, W340F; and 4, W356Y). The relative contribution of each tryptophan to actin fluorescence is given in Table 3.

TABLE 3 Contributions of tryptophans to the intrinsic fluorescence of actin

	Fluorescence decrease relative to wild-type actin (%)	
Actin	Decrease in Fig. 3	Average decrease
Wild-type	0	0
W79Y	1	1
W86F	16	11
W340F	33	37
W356Y	50	51

The individual contributions (%) of the four tryptophans to wild-type actin fluorescence are taken to be equal to the relative fluorescence decrease observed for each mutant, measured in the Ca^{2+}-G-actin form. Values for the averaged data represent six experiments and are normalized to 100%.

fluorescence spectra of the latter to be blue-shifted compared to the former. Given the small contributions of W79 and W86 to actin's fluorescence, the λ_{max} shifts caused by their substitutions would escape our detection in the available mutants. However, in agreement with their prediction, the emission spectrum of W79 actin is red-shifted (λ_{max} = 335 nm) relative to that of wild-type actin (Fig. 6).

It has been shown previously that the intrinsic fluorescence of actin is changed upon the transition from the Ca^{2+}- to Mg^{2+}-G-actin forms, and again as the protein polymerizes (Selden et al., 1994). Rabbit actin shows a small increase in fluorescence upon metal cation exchange, and a larger decrease due to its polymerization. This is also true for the yeast wild-type actin and for most of the mutants. However, it is apparent from the examples shown in Fig. 4 that the changes in spectra can be relatively small (especially, for instance, with metal cation exchange in the W356Y mutant). Consequently, a more convenient representation of the data is in the form of difference spectra (shown in Fig. 5) for cation exchange (upper, solid curves) and Mg^{2+}-G-actin polymerization by 4.0 mM magnesium chloride (lower, dashed curves). It is apparent that the Ca^{2+}-Mg^{2+} difference spectra are similar in all respects for wild-type and mutant actins, with the exception of the W356Y (Fig. 5f) and W79 (data not shown) mutants, which shows very little or almost no such fluorescence difference. This implies that it is mainly the environment around W356 that is affected by divalent cation exchange in actin.

The differences among the fluorescence difference spectra for actin polymerization are more subtle than those for cation exchange (Fig. 5). These difference spectra imply that more than one tryptophan contributes to fluorescence change upon actin polymerization as variations in the λ_{max} or peak size are detected for the W86F, W340F, and W356Y mutants. However, on the cautious side, these variations may well be within the resolution of our measurements.

Based on the results of the single tryptophan mutations, a protocol was devised to generate a trp-null mutant, with potentially useful double and triple mutants being generated en route. Given the relatively small contribution from W86 and an almost insignificant contribution from W79 to actin fluorescence, a double mutant containing these two residues was first constructed (W340F/W356Y). A triple mutant with the potentially fluorescently silent W79 remaining was then made; finally, a trp-null mutant was made. Although the final mutant actin supported yeast growth as the sole source of actin, repeated attempts to purify the trp-null protein proved unsuccessful. It appears unlikely that this protein would be unable to bind to the DNase column because of a specific damage to the DNase I binding loop in subdomain 2 of actin. However, it is possible that the trp-null actin is unstable when isolated, and that other cytoskeletal proteins expressed in yeast stabilize this actin in vivo.

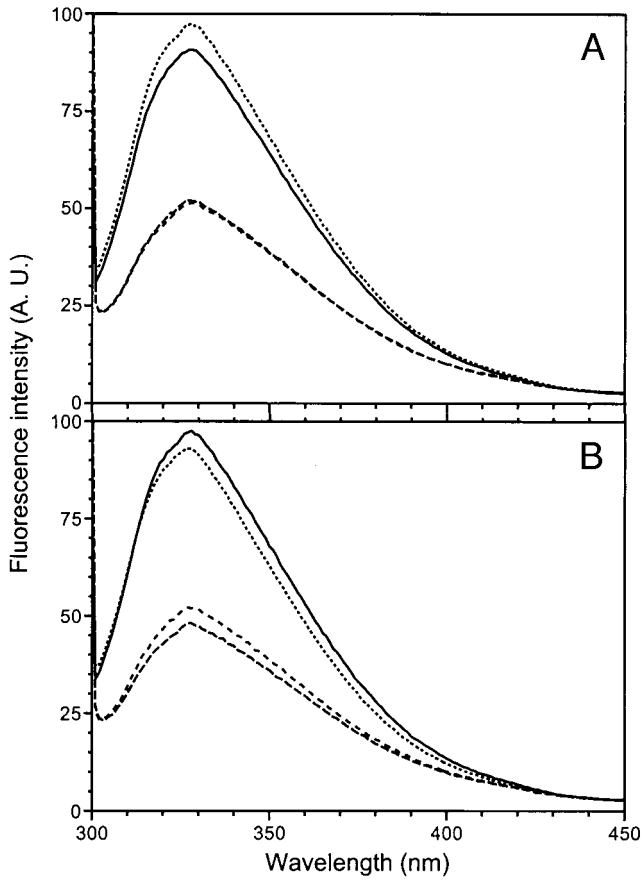

FIGURE 4 Tryptophan fluorescence changes associated with a Ca^{2+}/Mg^{2+} exchange and magnesium-induced actin polymerization. (*a*) Fluorescence emission of W79Y and W356Y actins in the Ca^{2+}- and Mg^{2+}-G-actin forms. For cation exchange, 4.0 μM actin with 20 μM Ca^{2+} was incubated with 100 μM EGTA and 100 μM Mg^{2+} for 6 min. W79Y Ca^{2+}-G-actin (*solid line*), W79Y Mg^{2+}-G-actin (*dotted line*), W356Y Ca^{2+}-G-actin (*dashed line*), W365Y Mg^{2+}-G-actin (*long dashed line*). (*B*) Fluorescence spectra of Mg^{2+}-G-actin and F-actin. For polymerization of G-actin (4.0 μM), magnesium concentration was increased from 100 μM to 4.0 mM. W79Y Mg^{2+}-G-actin, (*solid line*), W79Y F-actin (*dotted line*), W356Y Mg^{2+}-G-actin (*dashed line*), W356Y F-actin (*long dashed line*). The fluorescence data are shown in arbitrary units.

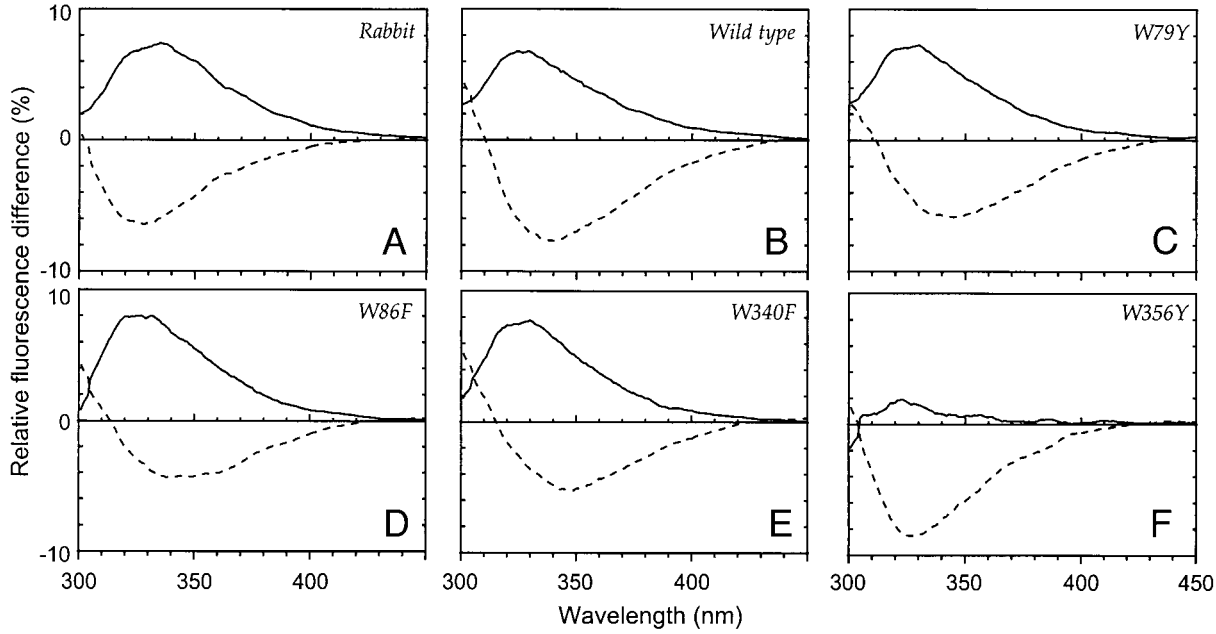

FIGURE 5 Fluorescence difference spectra (%) of (*A*) rabbit, (*B*) yeast wild-type, (*C*) W79Y, (*D*) W86F, (*E*) W340F, and (*F*) W356Y actins after metal cation exchange (*solid line*) and polymerization of G-actin (*dashed line*), as described in the legend to Fig. 4. The difference spectra were smoothed using the Savitsky-Golay algorithm over a 9-nm spread.

In contrast to the trp-null actin mutant, the W79 triple mutant, which also supports yeast growth, can be purified in sufficient yields to allow spectroscopic studies. Importantly, this mutant showed small fluorescence compared to the wild-type actin (Fig. 6). Although the fluorescence of W79 actin is greater (~12%) than that predicted from single tryptophan mutants (Table 3; ~1%), such differences could be ascribed either to some energy transfer between tryptophans (which is eliminated in W79 actin), or to minor local conformational changes in the vicinity of W79 residue, leading to a slightly less efficient quenching of its fluorescence in the W79 mutant.

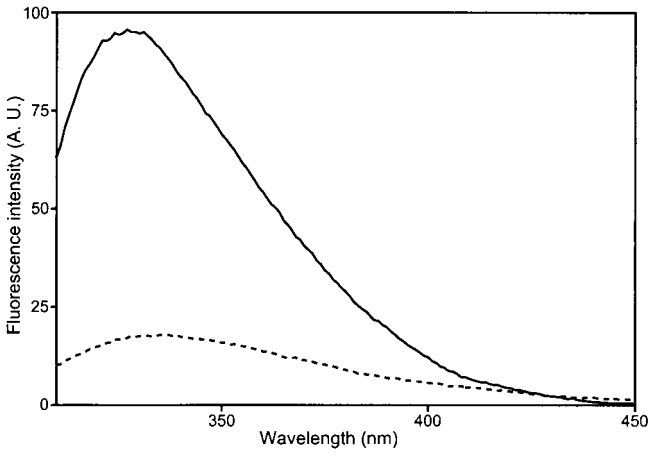

FIGURE 6 Fluorescence emission spectra of wild-type and W79 mutant actins. Experimental conditions are as stated in Fig. 3 legend. The spectra for wild-type (*solid line*) and W79 (*dashed line*) actins are shown in arbitrary units.

In summary, the four single tryptophan actin mutants studied in this work provide clues to the relative contributions of each tryptophan to the actin fluorescence and to the changes in its fluorescence as the protein undergoes conformational transitions. W356 has been linked to the cation exchange-induced fluorescence increase in actin, whereas two or three tryptophans appear to be involved in the polymerization-related quenching of actin fluorescence. The information on relative quantum yields of the four tryptophans in actin obtained here is particularly valuable because it enables measurements of fluorescence resonance energy transfer from these residues to extrinsic probes on actin or actin binding proteins. The availability of W79 mutant actin, with its small fluorescence and acceptable binding of myosin, opens attractive possibilities for probing the effect of actin on specific sites on S1 (and on other proteins) via tryptophan mutations at these sites (Yengo et al., 1999, 1998).

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REFERENCES

Atkins, W. M., P. S. Stayton, and J. J. Villafranca. 1991. Time-resolved fluorescence studies of genetically engineered Escherichia coli glutamine synthetase. Effects of ATP on the tryptophan-57 loop. *Biochemistry.* 30:3406–3416.

Burstein, E. A., N. S. Vedenkina, and M. N. Ivkova. 1973. Fluorescence and the location of tryptophan residues in protein molecules. *Photochem. Photobiol.* 18:263–279.

Cook, R. K., D. Root, C. Miller, E. Reisler, and P. A. Rubenstein. 1993. Enhanced stimulation of myosin subfragment 1 ATPase activity by addition of negatively charged residues to the yeast actin NH_2-terminus. *J. Biol. Chem.* 268:2410–2415.

Cooke, R. 1997. Actomyosin interaction in striated muscle. *Phys. Rev.* 77:671–697.

Crosbie, R. H., C. Miller, P. Cheung, T. Goodnight, A. Muhlrad, and E. Reisler. 1994. Structural connectivity in actin: effect of C-terminal modifications on the properties of actin. *Biophys. J.* 67:1957–1964.

Eftink, M. R., and C. A. Ghiron. 1976. Exposure of tryptophanyl residues in proteins. Quantitative determination by fluorescence quenching studies. *Biochemistry.* 15:672–680.

Eftink, M. R., and C. A. Ghiron. 1977. Exposure of tryptophanyl residues and protein dynamics. *Biochemistry.* 16:5546–5551.

Feng, L., E. Kim, W. L. Lee, C. J. Miller, B. Kuang, E. Reisler, and P. A. Rubenstein. 1997. Fluorescence probing of yeast actin subdomain 3/4 hydrophobic loop 262–274: actin-actin and actin-myosin interactions in actin filaments. *J. Biol. Chem.* 272:16829–16837.

Godfrey, J. E., and W. F. Harrington. 1970. Self-association in the myosin system at high ionic strength. I. Sensitivity of the interaction to pH and ionic environment. *Biochemistry.* 9:886–893.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163–168.

Kabsch, W., H. G. Mannherz, D. Suck, E. F. Pai, and K. C. Holmes. 1990. Atomic structure of the actin:DNase I complex. *Nature.* 347:37–44.

Kim, E., C. J. Miller, M. Motoki, K. Seguro, A. Muhlrad, and E. Reisler. 1996. Myosin-induced changes in F-actin: fluorescence probing of subdomain 2 by dansyl ethylenediamine attached to Gln-41. *Biophys. J.* 70:1439–1446.

Kim, E., M. Motoki, K. Seguro, A. Muhlrad, and E. Reisler. 1995. Conformational changes in subdomain 2 of G-actin: fluorescence probing by dansyl ethylenediamine attached to Gln-41. *Biophys. J.* 69: 2024–2032. .

Kron, S. J., T. Q. Uyeda, H. M. Warrick, and J. A. Spudich. 1991. An approach to reconstituting motility of single myosin molecules. *J. Cell Sci.* 14(suppl.):129–133.

Kuznetsova, I. M., O. Antropova, K. Turoverov, and S. Khaitlina. 1996. Conformational changes in subdomain I of actin induced by proteolytic cleavage within the DNase I-binding loop: energy transfer from tryptophan to AEDANS. *FEBS Lett.* 383:105–108.

Kuznetsova, I. M., T. A. Yakusheva, and K. K. Turoverov. 1999. Contribution of separate tryptophan residues to intrinsic fluorescence of actin: analysis of 3D structure. *FEBS Lett.* 452:205–210.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Li, J., R. Szittner, and E. A. Meighen. 1998. Tryptophan fluorescence of the lux-specific Vibrio harveyi acyl-ACP thioesterase and its tryptophan mutants: structural properties and ligand-induced conformational change. *Biochemistry.* 37:16130–16138.

Loewenthal, R., J. Sancho, and A. R. Fersht. 1991. Fluorescence spectrum of barnase: contributions of three tryptophan residues and a histidine-related pH dependence. *Biochemistry.* 30:6775–6779.

Lorenz, M., D. Popp, and K. C. Holmes. 1993. Refinement of the F-actin model against X-ray fiber diffraction data by the use of a directed mutation algorithm. *J. Mol. Biol.* 234:826–836.

Mårtensson, L. G., P. Jonasson, P. O. Freskgård, M. Svensson, U. Carlsson, and B. H. Jonsson. 1995. Contribution of individual tryptophan residues to the fluorescence spectrum of native and denatured forms of human carbonic anhydrase II. *Biochemistry.* 34:1011–1021.

Miki, M., S. I. O'Donoghue, and C. G. Dos Remedios. 1992. Structure of actin observed by fluorescence resonance energy transfer spectroscopy. *J. Mus. Res. Cell Motil.* 13:132–145.

Miller, C. J., T. C. Doyle, E. Bobkova, D. Botstein, and E. Reisler. 1996a. Mutational analysis of the role of hydrophobic residues in the 338–348 helix on actin in actomyosin interactions. *Biochemistry.* 35:3670–3676.

Miller, C. J., and E. Reisler. 1995. Role of charged amino acid pairs in subdomain-1 of actin in interactions with myosin. *Biochemistry.* 34: 2694–2700.

Miller, C. J., W. W. Wong, E. Bobkova, P. A. Rubenstein, and E. Reisler. 1996b. Mutational analysis of the role of the N terminus of actin in actomyosin interactions: comparison with other mutant actins and implications for the cross-bridge cycle. *Biochemistry.* 35:16557–16565.

Milligan, R. A. 1996. Protein-protein interactions in the rigor actomyosin complex. *Proc. Natl. Acad. Sci. USA.* 93:21–26.

Rayment, I., H. M. Holden, M. Whittaker, C. B. Yohn, M. Lorenz, K. C. Holmes, and R. A. Milligan. 1993. Structure of the actin-myosin complex and its implications for muscle contraction. *Science.* 261:58–65.

Rouviere, N., M. Vincent, C. T. Craescu, and J. Gallay. 1997. Immunosuppressor binding to the immunophilin FKBP59 affects the local structural dynamics of a surface beta-strand: time-resolved fluorescence study. *Biochemistry.* 36:7339–7352.

Schröder, R. R., D. J. Manstein, W. Jahn, H. Holden, I. Rayment, K. C. Holmes, and J. A. Spudich. 1993. Three-dimensional atomic model of F-actin decorated with Dictyostelium myosin S1. *Nature.* 364:171–174.

Selden, L. A., H. J. Kinosian, J. E. Estes, and L. C. Gershman. 1994. Influence of the high affinity divalent cation on actin tryptophan fluorescence. *Adv. Expt. Med. Biol.* 358:51–57.

Sherman, F. 1991. Getting started with yeast. *Methods Enzymol.* 194:3–21.

Sherman, F., and J. Hicks. 1991. Micromanipulation and dissection of asci. *Methods Enzymol.* 194:21–37.

Sheterline, P., J. Clayton, and J. Sparrow. 1995. Actin. *Protein Profile.* 2:1–103.

Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics.* 122:19–27.

Smith, C. J., A. R. Clarke, W. N. Chia, L. I. Irons, T. Atkinson, and J. J. Holbrook. 1991. Detection and characterization of intermediates in the folding of large proteins by the use of genetically inserted tryptophan probes. *Biochemistry.* 30:1028–1036.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

Szpikowska, B. K., J. M. Beechem, M. A. Sherman, and M. T. Mas. 1994. Equilibrium unfolding of yeast phosphoglycerate kinase and its mutants lacking one or both native tryptophans: a circular dichroism and steady-state and time-resolved fluorescence study. *Biochemistry.* 33: 2217–2225.

Weeds, A. G., and B. Pope. 1977. Studies on the chymotryptic digestion of myosin. Effects of divalent cations on proteolytic susceptibility. *J. Mol. Biol.* 111:129–157.

Wertman, K. F., D. G. Drubin, and D. Botstein. 1992. Systematic mutational analysis of the yeast ACT1 gene. *Genetics.* 132:337–350.

Yengo, C. M., L. Chrin, A. S. Rovner, and C. L. Berger. 1999. Intrinsic tryptophan fluorescence identifies specific conformational changes at the actomyosin interface upon actin binding and ADP release. *Biochemistry.* 38:14515–14523.

Yengo, C. M., P. M. Fagnant, L. Chrin, A. S. Rovner, and C. L. Berger. 1998. Smooth muscle myosin mutants containing a single tryptophan reveal molecular interactions at the actin-binding interface. *Proc. Natl. Acad. Sci. USA.* 95:12944–12949.