A Highly Conserved 3-Methylhistidine Modification Is Absent in Yeast Actin

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To identify a protein histidine methyltransferase from Saccharomyces cerevisiae, we examined purified actin for the presence of the highly conserved 3-methylhistidine residue at position 73 by amino acid analysis of the whole protein and by amino acid analysis and mass spectrometry of the corresponding tryptic fragment. Surprisingly, we found that His-73 is not modified. A similar lack of modification was also found in actin from the yeast Candida albicans, while rabbit muscle actin revealed the expected 3-methylhistidine residue. Phylogenetic analysis of actin sequences suggests that this modification was introduced in evolution after the divergence of yeast from higher eukaryotic organisms, including unicellular eukaryotes such as Acanthamoeba, Dictyostelium, and Physarum, whose actins contain 3-methylhistidine. Our methodology for the analytical determination of 3-methylhistidine in actin offers an improved approach for investigating histidine methylation in proteins. © 1999 Academic Press

A histidine residue at position 73 is conserved in all known actins. With the single exception of the amoeba Naegleria gruberi actin, this histidine residue is posttranslationally modified to 3-methylhistidine (1–3). This unusual modification is also found in some striated myosin isoforms (4, 5). The functional significance of this modification of actin is unknown. Clearly, it is not required for Naegleria. When actin His-73 is replaced using site-directed mutagenesis, the isotopically labeled mutant proteins readily copolymerize with carrier wild-type actin (6). However, in this system, it is possible that defects in the small amount of labeled mutant actin could be compensated for by the much larger amount of unlabeled wild-type actin used in the copolymerization experiment. Recently, we have replaced this histidine residue in yeast actin with five other residues and have shown that the resulting actins are compatible with yeast viability (P. A. Rubenstein, unpublished data). The availability of these actin mutants will allow us to test the necessity of histidine at the position 73 in a homogeneous preparation, but it will not allow us to assess the contribution of the methyl group per se.

Although neither the methylation nor the histidine itself is required for viability, at least in yeast, the presence of this modified residue may still confer some quantitative advantage on actin function. The imidazole of this residue lies in actin’s subdomain 2 near the subdomain 1/2 interface on the exterior of the protein blocking a channel that allows water to enter the interior of the protein (7). From these data it has been suggested that the methylhistidine side chain, by interacting with phosphate ion released following hydrolysis of the actin-bound ATP during polymerization, may play an important role in preventing the facile exit of the phosphate through this channel (8). Since the stability of the actin filament is controlled by the state of the nucleotide bound to the monomer in the filament, such a role for the methylhistidine would maintain the actin filament for an extended period of time. This role for the methylhistidine, if true, would thus have a major impact on actin cytoskeletal dynamics within the cell.

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Despite the widespread occurrence of this modified residue in almost all actins, little if anything is known about the manner in which the methylation occurs. This is due in large part to the lack of a convenient assay and to the absence of an experimental system in which actin methylation can be prevented. Although Raghavan et al. (9) demonstrated a His-methylating enzyme in a mammalian cell extract using a peptide substrate, it is not clear that this activity can methylate the conserved histidine residue in actin.

We have been interested in identifying novel S-adenosylmethionine (AdoMet)\(^2\)-dependent methyltransferases in Saccharomyces cerevisiae, particularly enzymes that are involved in posttranslational modifications of proteins (10). Yeast is an ideal organism for such a search. The complete elucidation of its genome sequence allows one to identify potential candidates for such enzymes by searching for open reading frames with sequence motifs similar to those found in AdoMet-utilizing enzymes. Yeast is genetically manipulatable and amenable to use for enzyme purification, and its intact cells take up AdoMet so that methyl-acceptors can be radiolabeled. In this work, we focused on 3-methylhistidine formation in yeast actin. However, we found that this modification was absent in both S. cerevisiae actin as well as actin from the unrelated yeast Candida albicans.

**MATERIALS AND METHODS**

**Actin purification.** Rabbit muscle actin was a generous gift from Dr. Emil Reisler at UCLA. The site-directed mutagenesis procedure of Feng et al. (11) was used to generate a mutant S. cerevisiae strain carrying only the H73A actin. Details of the cell's phenotype and the behavior of the actin in vitro will be described elsewhere. Actin was isolated from extracts of mutant and wild-type S. cerevisiae and from C. albicans by a combination of DNase I affinity chromatography, DEAE-cellulose chromatography, and a cycle of actin polymerization/depolymerization as described previously (12).

**Amino acid analysis of in vivo labeled yeast extracts.** Intact yeast cells were labeled with [methyl-\(^3\)H]AdoMet and protein hydrolysates were analyzed on a column of Beckman AA-15 sulfonated polystyrene resin as described previously (10).

Fractionation of tryptic peptides of actin by HPLC. Purified actin (about 500 \(\mu\)g, 12 nmol) was denatured in 500 \(\mu\)l of 8 M urea for 1 h at 37°C. The solution was then diluted to give final concentrations of 2 M urea, 1.5 mM Tris–HCl pH 7.5, and 3 mM CaCl\(_2\). Trypsin (25 \(\mu\)g, Sigma type XIII, N-tosyl-l-phenylalanine chloromethyl ketone-treated) was added and the mixture was incubated at 37°C for 4 h. The entire digest was injected onto a C18 reverse-phase column (Microsorb-MV, 5 \(\mu\)m, 4.6-mm inside diameter, 250-mm length, Rainin) that was equilibrated in solvent A (0.1% trifluoroacetic acid in water) and eluted at room temperature at a flow rate of 1 ml/min with a linear gradient of solvent B (0–100%, 0.1% trifluoroacetic acid in acetonitrile) over 60 min. Eluant absorbance was monitored at 214 and 295 nm. Fractions were collected manually.

**RESULTS**

To study actin methylation in yeast, we incubated intact S. cerevisiae cells with [methyl-\(^3\)H]AdoMet to label methylated cell components. Total cellular protein hydrolysates were analyzed by amino acid analysis to compare the methylated amino acid residues of wild-type yeast and a strain in which the actin gene is mutated to convert the conserved histidine residue at position 73 to an alanine residue to prevent methylation (Fig. 1). There was no reduction in the amount of radioactivity that eluted at or near the position of 3-methylhistidine in the hydrolysate of the mutant which contained Ala-73 actin. These results suggest that either the fraction of the total yeast 3-methylhistidine in actin is minimal or that yeast actin is not methylated at the conserved histidine.

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\(^2\) Abbreviations used: AdoMet, S-adenosyl-l-methionine; [methyl-\(^3\)H]AdoMet, S-adenosyl-l-[methyl-\(^3\)H]methionine.
To directly examine the methylation of this histidine residue in yeast actin, we carried out amino acid analyses of the purified actin from *S. cerevisiae* and from rabbit muscle actin as a control. We optimized the chromatographic conditions to separate the o-phthalaldehyde derivatives of 3-methylhistidine from those of the other amino acids present in the hydrolysate. An elution system was developed in which the fluorescent derivative of 3-methylhistidine elutes as a single symmetrical peak at 9.5 min, baseline resolved from the histidine peak at 8.0 min and from the arginine peak at 13.0 min (Fig. 2A). The hydrolysate of purified rabbit actin showed a peak at 9.5 min retention time, corresponding to 3-methylhistidine as expected (Figs. 2A and 2B, trace A), but no peak was evident in the *S. cerevisiae* actin hydrolysate (Fig. 2B, trace B). The hydrolysate of purified actin from *C. albicans* also did not contain a peak eluting at the retention time corresponding to 3-methylhistidine (Fig. 2B, trace C). These results strongly suggest that actins from these fungi are not modified by histidine methylation.

To confirm unambiguously that His-73 is not methylated in actins of *S. cerevisiae* and *C. albicans*, we isolated the actin tryptic fragments containing His-73 from the purified proteins. Although the HPLC separation of trypsinized actin from mammalian brain cells has been reported in the literature (15), the assignment of the HPLC retention time for each tryptic fragment has not been done. Therefore, we exploited the presence of a single conserved Trp residue in the predicted 69–84 tryptic peptide containing the His-73 residue from each of the species. The amino acid sequences of actins from yeast and rabbit predict a total of only three Trp-containing tryptic peptides; one is expected to elute earlier than the His-73 containing peptide, and the third fragment is expected to elute later (16). Using an absorbance detector at 295 nm to specifically monitor tryptophan-containing peptides, we were able to detect two main peaks for each trypsinized actin (Fig. 3). The predicted third fragment was not detected under these conditions. The second peak had the expected elution time for the 69–84 fragment, and it was collected and analyzed by mass spectroscopy and amino acid analysis as described below.

The electrospray mass spectra of the HPLC-purified putative actin 69–84 fragments from rabbit, *C. albicans*, and *S. cerevisiae* all revealed signals for the doubly and triply charged molecules (data not shown). The molecular weight calculation based on these signals for the rabbit peptide agreed within experimental error with that calculated for the 69–84 tryptic fragment in which His-73 is substituted with methylhistidine (Table I). In contrast, the spectra of the *S. cerevisiae* and the *C. albicans* fractions were consistent with peptides containing an unmodified histidine residue (Table I). For rabbit actin, we saw no signal for the corresponding nonmethylated tryptic fragments, and the yeast samples gave no signal for a methylated fragment. The limits of detection were estimated at less than 5% of the main signals.

We next confirmed the presence or absence of methylhistidine in the purified tryptic fragments by the amino acid analysis system described in the legend to Fig. 2. As expected, a symmetrical peak with elution time of 9.5 min corresponding to 3-methylhistidine was detected only from the purified rabbit tryptic fragment (Fig. 4). For each eluted tryptic fragment, the amount of 3-methylhistidine was calculated with respect to the peak for aspartate and glutamate (not shown). We found 1.05 mol of 3-methylhistidine per mole of purified rabbit tryptic fragment, but less than 0.007 mol of 3-methylhistidine per mole of the *S. cerevisiae* and *C. albicans* tryptic fragments.

The tryptic fragments were further analyzed by MS/MS to prove that the observed molecular mass difference between the rabbit tryptic fragment and those of the yeast was the result of an extra methyl group on the histidine residue at position 73. The MS/MS spectra collected from collisionally activated dissociation of each doubly protonated molecule, revealed a series of fragment ions (Fig. 5). The majority...
of these could be assigned as b- or y-type ions on the basis of their observed m/z values by comparison to the predicted array of fragments for each putative peptide. Both y- and b-type ions are formed by cleavage of the amide bonds; charge retention on the N-terminal fragment gives rise to b-type ions, and charge retention on the C-terminal fragment gives rise to y-type ions. In particular, each spectrum unequivocally revealed b4/b5 and y11/12 ion pairs which spanned the putative His-73 residue in S. cerevisiae and C. albicans and the methylhistidine in rabbit (Fig. 5). These results clearly demonstrate that His-73 is methylated in the rabbit tryptic fragment and that it is not methylated in that of either S. cerevisiae or C. albicans.

We were initially puzzled that some actins from unicellular eukaryotes are methylated (i.e., Dictyostelium discoideum (17), Physarum polycephalum (18), and Acanthamoeba castellanii (19)) and that some are not (i.e., Naegleria gruberi (20), S. cerevisiae, and C. albicans (this work)). To determine if there might be an evolutionary basis for the difference in the methylation between these two groups of actins, a phylogenetic tree was constructed using the primary sequences of actins from several species in which absence or presence of methylation of His-73 has been reported. The results, shown in Fig. 6, demonstrate a clear phylogenetic demarcation between those organisms that are methylated and

**FIG. 2.** Amino acid analysis of 3-methylhistidine in purified actin hydrolysates. (A) o-Phthalaldehyde-derivated standard amino acids (Pierce Standard H, 100 pmol) with or without 3-methylhistidine (150 pmol) and the acid hydrolysate of rabbit muscle actin were chromatographed on a reverse-phase HPLC column as described under Materials and Methods. The elution positions for the derivatized amino acids are indicated by arrows and 3-methylhistidine is labeled as 3-MeHis. In this system, aspartate and glutamate coelute, serine and threonine coelute, histidine gives two peaks, and the hydrophobic amino acids are retained. (B) Enlarged portions of the chromatographs for derivatized hydrolysates of purified actin from rabbit (trace A), S. cerevisiae (trace B), and C. albicans (trace C).
those that are not. This tree is consistent with the idea that the methylation of His-73 appeared in evolution after the separation of yeast from Dictyostelium, Physarum, Acanthamoeba, and higher organisms. The tree also agrees with previous reports that Dictyostelium is more closely related to mammalian cells than is yeast (21).

**FIG. 3.** HPLC fractionation of the trypsin digest of purified actins. Actins from rabbit skeletal muscle, S. cerevisiae, and C. albicans were digested with trypsin, and the resulting peptides fractionated using reverse-phase HPLC as described under Materials and Methods. The HPLC eluate was monitored at 214 and 295 nm. The tryptic fragment 69–84, identified by electrospray mass spectrometry (Table 1) and amino acid analysis (Fig. 4), is indicated by an asterisk.

**FIG. 4.** Amino acid analysis of 3-methylhistidine in purified tryptic peptides. HPLC-purified tryptic peptide 69–84 from rabbit, S. cerevisiae, and C. albicans was acid hydrolyzed, o-phthalaldehyde derivatized, and eluted on a reverse-phase HPLC column as shown in Fig. 2. 3-Methylhistidine is labeled as 3-MeHis.

**DISCUSSION**

Despite the high conservation of the uncommon 3-methylhistidine residue at position 73 in actin, we have documented the absence of this posttranslational modification in two distantly related yeast species by amino acid analysis and mass spectrometry. We have shown that there appears to be a clear evolutionary demarcation among species that do or do not methylate actin His-73 residues, suggesting that this modification was introduced after the appearance of unicellular eukaryotes. Our earlier work demonstrated another type of posttranslational processing in actins from cells ranging from Dictyostelium to mammals. This modification involves N-terminal processing in which one or two amino acids are removed singly from the N-terminus following acetylation as N-acetylamino acids until an acidic residue is reached. Acetylation of this residue...
produces the final N-acetylated amino terminus of the protein (22–25). Interestingly, this modification also does not occur in the yeasts *S. cerevisiae* and *C. albicans*, nor the yeast *Schizosaccharomyces pombe* or the filamentous fungus *Aspergillus nidulans* (26). When we examined Acanthamoeba actin, we found that processing did not occur both because the pre-actin was not a substrate and because the organism itself did not possess processing activity (27). Although we did not study the processing in *Naegleria*, this particular type of processing cannot occur in this organism since the coding sequence for its N-terminus includes 10 additional amino acids which are not found in its mature form of the protein (27). Thus, our demonstration of the lack of histidine methylation would be the second post-translational modification that separates the actins of this older group of protists from their more evolutionarily advanced counterparts. However, since Acanthamoeba actin shows methylation of His-73 and not the N-terminus processing, these two different modifications most likely did not arise in parallel.

The methodology reported here for determination of methylhistidine in actin offers a significant advantage over previous approaches (20, 29, 30).

**FIG. 5.** MS/MS spectra of purified peptides. MS/MS spectra derived by collisionally activated dissociation of the doubly charged parent (P) for rabbit (A), *S. cerevisiae* (B), and *C. albicans* (C) purified tryptic fragment 69–84. Ions assignments (y and b) were made after comparison with the array of ions predicted for each peptide and are shown for a few crucial ions as observed m/z with calculated m/z in parentheses. The sequence for each tryptic fragment along with some of the b and y ion fragmentation is shown as an inset.

![Phylogenetic tree](image)

**FIG. 6.** Phylogenetic tree. Actin sequences were obtained from GenBank. Complete sequences of actin were aligned by the clustal method using the multiple sequence alignment program MegAlign (31). A phylogenetic tree was constructed by applying a neighborhood-joining method to the distance and alignment data (32). The actin sequence of *N. fowleri* was used in place of *N. gruberi*, which was not available. Species labeled with an asterisk contain 3-methylhistidine in their actin.
LACK OF METHYLATION OF YEAST ACTIN

dehyde derivative of methylhistidine can be clearly resolved chromatographically from that of other amino acids while taking advantage of the sensitivity of the fluorescent labeling. We were able to detect less than 1 mol % 3-methylhistidine in yeast actin, a much lower limit than the 20 mol % reported previously in N. gruberi (20). Whereas previous methods required a minimum of 500 μg of actin (30), just 100 μg of actin was sufficient to quantify 3-methylhistidine in this study.

Because the presence of the methyl group on His-73 changes the pKₐ of the imidazole, methylation could play an important role quantitatively in ionic processes such as interaction with the phosphate ion released following the hydrolysis of ATP within the cleft of the protein (7, 8). Likewise, methylation might modulate the ionic interaction with one or more of many actin binding proteins that interact with the actin filament between adjacent monomers in the part of the protein where His-73 is located. The extra hydrophobicity and bulk of the methyl group could also play a role in these processes. We simply do not have the answers to these questions yet because we have not had access to the same actin in the methylated vs the nonmethylated state.

The demonstration that yeast does not methylate actin now allows the possibility of testing these theories directly. Aspenstrom and Karlsson (28) have devised an actin expression system in yeast in which chicken nonmuscle β-actin is coexpressed with the endogenous yeast actin, and the two actins are separated chromatographically following cell lysis. This avian actin has a structure identical to β-nonmuscle actin from mammalian sources. Using this system, one could now compare the behavior of the β-actin isolated from yeast with that isolated from either avian or mammalian tissue to address the role of this modification in different in vitro assays. The finding that yeast actin is not modified also suggests that it may be useful as a methyl-acceptor substrate to identify the actin methyltransferase in higher organisms. In summary, our demonstration of the lack of actin histidine methylation in yeast may be a crucial step in leading to a better understanding of the role of this modification on actin function.

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