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CARBOXYL GROUP REACTIVITY IN ACTIN

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INTRODUCTION

Actin is the principal protein of the thin filament of muscle and is also the major component of microfilaments. At low salt concentrations, actin exists in solution as a globular monomer (G-actin) of M_r=42,000. It has a single polypeptide chain of 375 residues and a bound metal-nucleotide (Mg^{++} or Ca^{++}-ATP or ADP). In physiological ionic strength solution, the actin monomers polymerize, forming fibrous (F-) actin which is a two stranded helical "rope". G-actin has been crystallized as a 1:1 complex with DNase I (Suck et al., 1981) or with profilin (C. Shutt and U. Lindberg, pers. commun.), and its structure is being studied by x-ray crystallography. These studies are expected to lead to an understanding of the structure of the actin monomer, but they probably will not lead directly to an understanding of the organization of the actin monomers within F-actin. It is known that each monomer interacts with its neighbors (above and below), being displaced by about 5.5 nm along the long axis, and rotated by just over 180°. It is essential to know exactly which parts of the surface represent the points or regions of interaction between adjacent actins, and one way to establish the orientation of the actins is to measure the distance between an amino acid sidechain in one actin and a different sidechain in an adjacent actin, by chemical crosslinking. Such information, in conjunction with an x-ray derived structure, may help to

establish the complete structure of F-actin.

We have recently (Elzinga and Phelan, 1984) described an experiment in which the reagent p-phenylenedimaleimide (Knight and Offer, 1978) was shown to crosslink Cys-374 from one monomer of F-actin and Lys-191 from an adjacent actin. This result must mean that these sidechains lie 1.2-1.4 nm (the length of the crosslinker) apart in F-actin. Our efforts to introduce and characterize additional intermolecular crosslinks have been hampered by an inability to attach bifunctional crosslinkers selectively to different sites in actin. It should be noted that Cys-374 is by far the most reactive sidechain in actin (Elzinga and Collins, 1975), and the crosslink mentioned above results from an initial rapid reaction of one end of the di-maleimide with Cys-374, followed by a slower reaction of the immobilized reagent with Lys-191, which is probably the only reactive sidechain in the vicinity. In an effort to introduce bifunctional reagents into additional sites in actin, we are studying the specificity of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) mediated binding of amino-group-containing reagents into the carboxyl groups of actin. Hoare and Koshland (1967) showed that, at pH 4.75 and in denaturing solvents, the carboxyl groups could be quantitatively reacted with the nucleophile glycine methyl ester in the presence of EDC. The pH dependence of this reaction has not been studied systematically, but it is known that EDC will induce crosslinking of proteins at pH's near neutrality (Mornet et al., 1981), presumably through reaction of lysine amino groups with the carboxylic acid sidechains of glutamic acid or aspartic acid. In the work reported here, we have examined the specificity of EDC-mediated incorporation of glycine ethyl ester (GEE) into F-actin at pH 7.25.

METHODS

Actin was prepared from an acetone powder of rabbit skeletal muscle. To 25 mL of a solution of F-actin (2 mg/mL in 2 mM tris, pH 7.25, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.1 M NaCl), ^{14}C glycine ethyl ester (at pH 7.25) was added to 14 mM and incubated at 22° for 10 min. Sufficient 1 M EDC in water was added to bring the concentration to 10 mM. After 60 min. the reaction was quenched by addition of solid DTT to 10 mM. The solution was dialyzed exhaustively against H_2O, and the actin was then

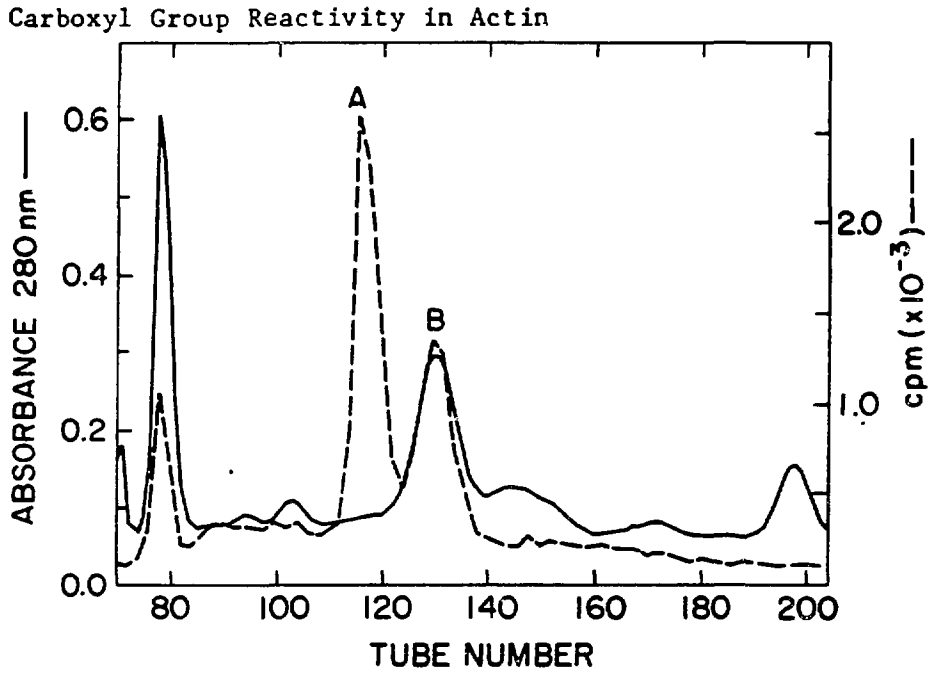

Fig. 1. Gel filtration of an arginine-specific tryptic digest of GEE-labeled actin. Fractions (5 mL) were collected at a flow rate of 15 mL/hr at 20°.

denatured and digested as described previously (Elzinga and Phelan, 1984). The procedure included carboxymethylation of the -SH groups, succinylation of the lysines, digestion (at the arginines) by trypsin, and gel filtration on Sephadex G-50. The elution profile for the digest is shown in Fig. 1. The major radioactive fractions (A and B) were collected and subjected to HPLC analysis, as illustrated in Fig. 2. While peak B yielded a single major radioactive peptide, the peptides in peak A did not behave well. This fraction was then digested with chymotrypsin (1% by weight) under conditions described earlier (Collins and Elzinga, 1975), and passed over a column of Dowex 50-X2.

RESULTS

Based upon amino acid analysis and total radioactivity measurements of the actin after treatment with GEE in the presence of EDC, it was estimated that 3-4 moles of GEE were incorporated per mole of actin. Most of the radioactivity emerged in two peaks from the G-50 column. About 10% of the counts emerged at the

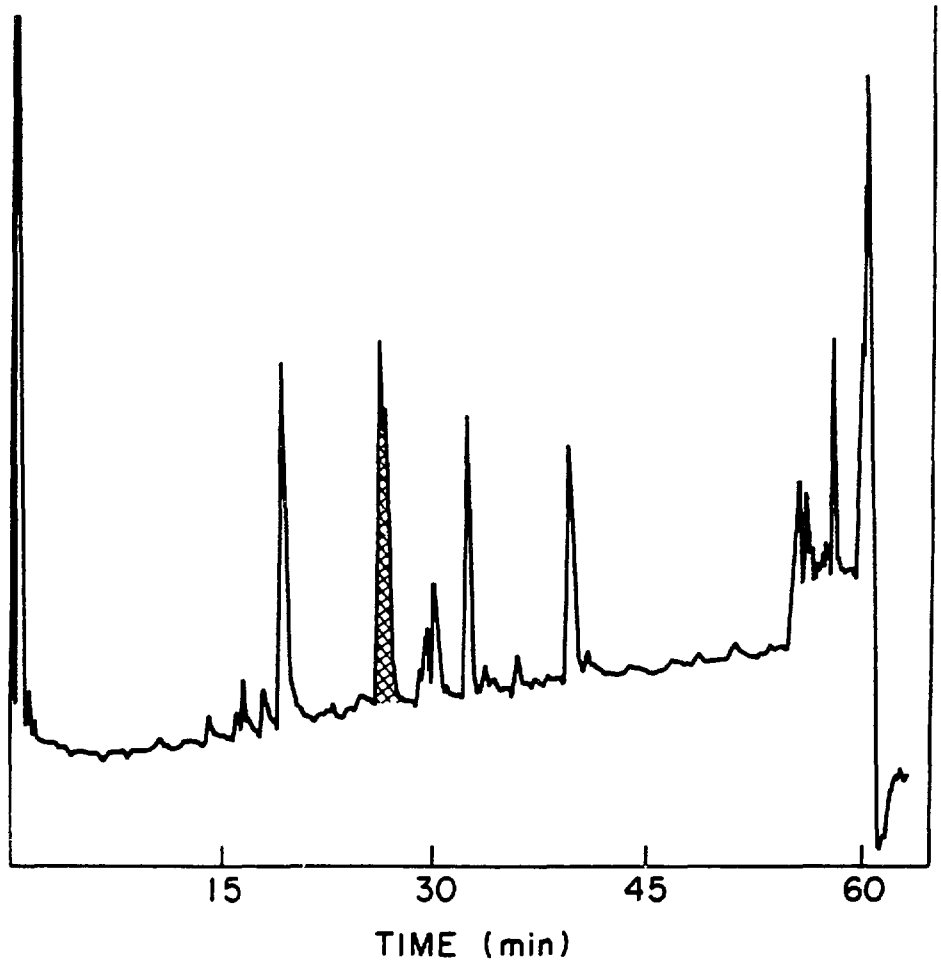

Fig 2. HPLC of Peak B of Fig. 1. About 10 nmol of the peptide mixture in 50 µL of 50% formic acid were applied. The instrument used was a Hewlett-Packard 1084 B and the column a Hibar EC RP-18 (10 µm; 4 x 150 mm). It was run at 1 mL/min. Solvent A was 0.1% TFA in H_2O, solvent B was 0.1% TFA in acetonitrile, the gradient was 10-45% B over 55 min. The crosshatched peak emerged at 27 min.

"breakthrough" but, since none of the authentic "arginine peptides" emerge there, it was assumed that the breakthrough peak contained only undigested an/or aggregated material.

Carboxyl Group Reactivity in Actin

AMINO ACID	residues 1-8	residues 96-116	residues 96-116 (leading edge)	residues 96-116 (trailing edge)
Lysine		1.05 (1)	1.08 (1)	1.04 (1)
Histidine		1.10 (1)	1.09 (1)	1.00 (1)
Arginine		0.95 (1)	0.97 (1)	1.01 (1)
Aspartic Acid	1.96 (2)	2.10 (2)	1.98 (2)	2.18 (2)
Threonine	1.86 (2)	1.94 (2)	1.74 (2)	2.12 (2)
Glutamic Acid	2.09 (2)	3.05 (3)	3.03 (3)	3.08 (3)
Proline		3.90 (4)	4.10 (4)	3.94 (4)
Glycine	2.75	0.80	1.03 (1)	0.19 (0)
Alanine	1.05 (1)	3.04 (3)	2.86 (3)	2.85 (3)
Valine		0.91 (1)	0.98 (1)	0.93 (1)
Leucine	1.04 (1)	2.96 (3)	2.91 (3)	2.82 (3)

Table 1. Amino acid composition of GEE labeled peptides. In each case, the observed glycine was released from GEE bound to sidechain carboxyl groups.

Peak A (Fig. 1) was subjected to HPLC under conditions described below for Peak B. A fraction of the peak that would represent about 10 nmol was applied, and among the peaks obtained none was radioactive. The labeled peptides apparently did not chromatograph well in this system. 50% of Peak A was then digested with 1% by weight of chymotrypsin in 0.5% NH_4HCO_3, pH 8.0, 20°, 4 hr. The digest was lyophilized, dissolved in 5 mL of 5% acetic acid and applied to a 1 x 10 cm column of Dowex 50-X2 equilibrated with 5% acetic acid. Essentially all of the radioactivity emerged unritarded from the column, suggesting that it was bound to a peptide that was devoid of positive charges. The radioactive peak was collected, and amino acid analysis of a small fraction of it gave the composition shown in column 1 of Table 1. The composition corresponded exactly to that of residues 1-8 of actin, plus 2.75 glycines. The amino terminus of actin is acetylated and the peptide therefore has no positive charges. The first four residues have carboxylic acid sidechains, and apparently at least three of them are fully or partially labeled with GEE. It is not possible

to determine the locations of the GEE's by direct sequencing because the NH_2-terminus of the peptide is blocked. It seems likely that the 2.75 moles of GEE are distributed among all four of the carboxylic acids, with a fraction of each being modified. Mass spectrometry (Hunt et al., 1986) may permit an unambiguous assignment of the sites of labeling.

Peak B (Fig. 1) was analyzed by HPLC (see Fig. 2), and the radioactivity was concentrated in one region whch appeared to be a double peak. Its amino acid composition (column 2 of Table 1) coincided with that of residues 96-116 of actin, plus 0.8 residues of glycine. This peptide was sequenced in both the Beckman 890 C and the Applied Biosystems 470 A instruments. In both runs, the radioactivity was found in step 5, corresponding to Glu-100 of actin. Additional evidence that Glu-100, and not Glu-99 or Glu-107, was labeled were the appearance of a new pth-aa peak at step 5, presumably the pth-derivative of the Glu-GEE adduct, and the appearance of both Glu and Gly upon amino acid analysis of an acid hydrolysis of step 5, while steps 4 and 12 released only Glu.

In amino acid analysis of peptide 96-116, only 0.8 residues of Gly were seen, suggesting that the reaction of GEE with Glu-100 was less than quantitative. The cross-hatched peak in Fig. 2 appeared to be a double peak, and the leading and trailing edges of the peak were collected and analyzed separately. The compositions are given in columns 3 and 4 of Table 1, and show that the leading edge has about 1 mole of Gly per mole of peptide, while the trailing edge has 0.19. It seems likely that this is contamination from the leading edge peptide, and that the trailing edge contains unmodified peptide. The composition of the total peak, with 0.8 Gly, probably indicates that Glu-100 is 80% reacted with GEE under the conditions employed here.

DISCUSSION

While the earlier work of Hoare and Koshland (1967) showed that the carboxyl groups of proteins could be quantitatively coupled to amino groups at pH 4.75 in the presence of EDC and a denaturing agent, the work presented here indicates that under milder conditions the modification of sidechain carboxyls can be limited and somewhat

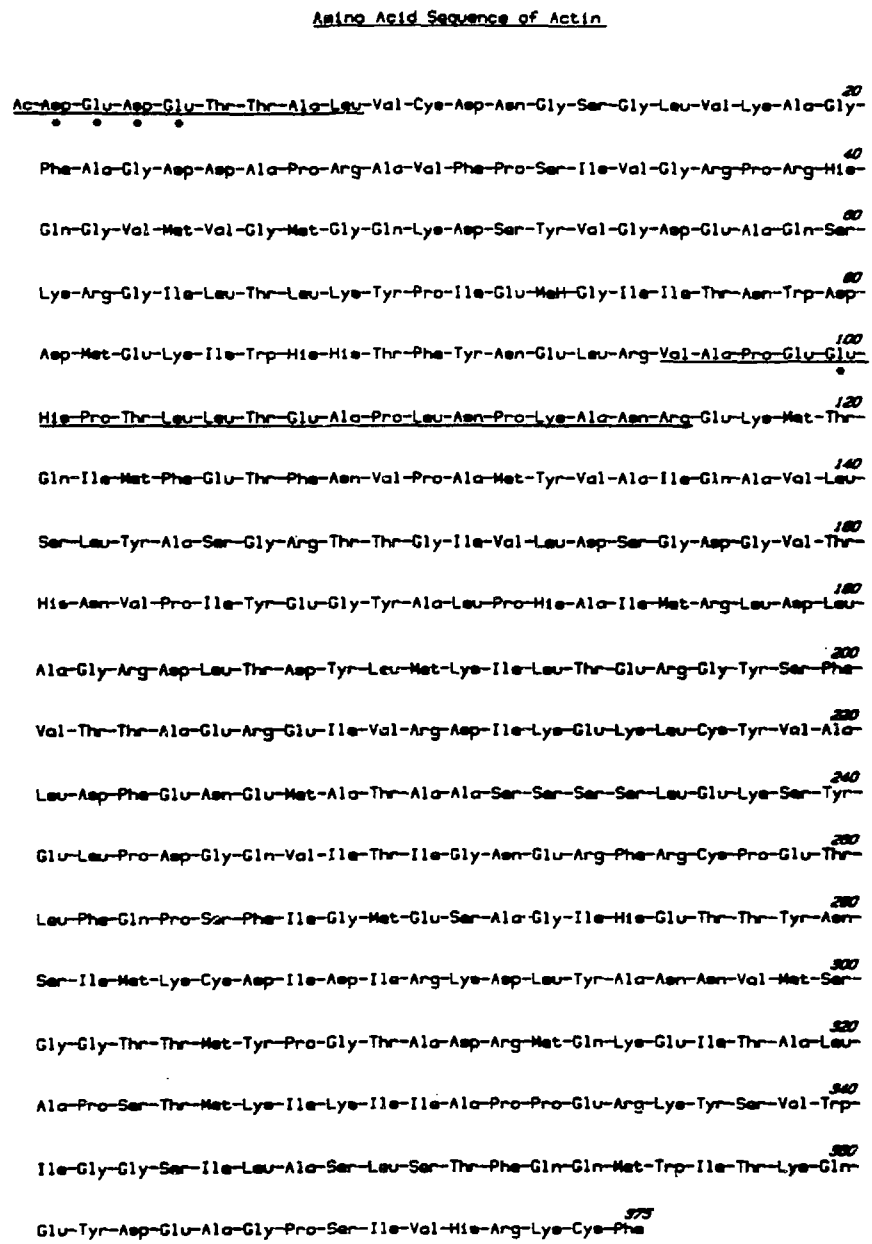

Fig. 3. The amino acid sequence of rabbit skeletal muscle actin. The labeled peptides are underlined, and the labeled residues are starred.

specific. Most of the incorporated GEE is apparently bound to five carboxyls. The total GEE incorporated was 3-4 moles/mole of protein as measured by an increase in Gly upon acid hydrolysis and amino acid analysis, as well as total radioactivity. 3.55 residues were found in peptides, 2.75 bound to residues 1-4, and 0.8 bound to Gly-100. Furthermore, the only radioactive peptides that were found upon chromatography were those whose compositions are given in Table 1, and it therefore seems likely that essentially all of the incorporation was into these positions; these locations are summarized in Fig. 3.

The carboxyls at the amino terminus of actin have been implicated in EDC mediated crosslinking to myosin and to actin-binding proteins (Sutoh, 1982); this has been taken as evidence that they are located physically near to the sites of protein-protein interaction. Since these groups are particularly reactive with GEE, it seems possible that they are intrinsically highly reactive, and that this contributes to their involvement in intermolecular crosslinking. Glu-100 has not previously been implicated in any actin functions; it is presumably on the surface, readily accessible to the reagents GEE and EDC, but it is not clear why it, and not the other carboxyls in the vicinity, Glu-99 and Glu-107, accepts GEE.

The overall goal of this work is to identify new sites at which to attach bifunctional crosslinking reagents; the results presented here suggest that EDC-mediated incorporation into carboxyls can be very specific. We are now attempting to incorporate reagents that possess both an amino group and a benzophenone moeity into these sites of actin, since the benzophenone can potentially bind to an adjacent actin molecule after photoactivation.

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