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F-actin is intermolecularly crosslinked by *N,N'-p*-phenylenedimaleimide through lysine-191 and cysteine-374

(muscle/thin filament/polymerization/amino acid sequence)

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ABSTRACT **The bifunctional reagent *N,N'-p*-phenylenedimaleimide (PDM) is being used in an attempt to measure distances between specific side chains in adjacent monomers within F-actin. [^{14}C]PDM was synthesized and was used to crosslink F-actin. Uncrosslinked actin was removed by gel filtration, and, from an arginine-specific tryptic digest of the covalently crosslinked dimers and higher oligomers, one radioactive crosslinked peptide was obtained in high yield. Amino acid composition and sequence analysis indicated that it comprises residues 184–196 of one monomer and 373–375 of an adjacent actin molecule, bridged by PDM through Cys-374 and Lys-191. Thus, these groups are shown to be 1.2–1.4 nm apart in adjacent actin monomers in F-actin. This information may be crucial in establishing the orientation of actin monomers within F-actin.**

F-actin is the major constituent of the thin filaments of muscle and a key component of the cytoplasmic matrix of cells. It is assembled from identical G-actin subunits, each of which is a protein having a single polypeptide chain of 375 amino acid residues folded, together with a bound ADP and divalent cation (Ca^{2+} or Mg^{2+}), into a roughly globular molecule. G-actin polymerizes, forming F-actin, under physiological salt conditions. In striated muscle, most of the actin exists as F-actin, whereas in other cells there is a dynamic equilibrium between polymerized and nonpolymerized actin, which is modulated by a variety of factors. In order to understand the details of actin polymerization and function, it is important to describe the structure of F-actin. This will require both a resolution of the three-dimensional structure of the actin monomer and an explanation of the orientation of the monomers within F-actin. The structure of the actin monomer is being investigated by x-ray crystallographic methods (1, 2), and it seems likely that atomic resolution will soon be achieved. Three-dimensional reconstruction of electron microscopic images of F-actin also has given some insight into the shape of the actin monomer within F-actin (3–5), and the combination of this information with a high-resolution picture of the actin monomer may lead to an understanding of the orientation of the actin monomer within the filament. On the other hand, the technique of three-dimensional reconstruction may not be capable of sufficiently high resolution to orient the monomers unequivocally; therefore, it is important to have independent information about the geometrical relationships between adjacent actin monomers. Chemical crosslinking can in principle provide such information, and Knight and Offer (6) have shown that F-actin can be crosslinked by the reagent *N,N'-p*-phenylenedimaleimide (PDM). They reported that PDM reacts first with a cysteine residue and that crosslinking results from a subsequent reaction with a lysine residue in a neighboring actin. In this paper we describe experiments in which we demonstrate that in PDM-crosslinked F-actin, the sulfhydryl group that is near the COOH terminus of rabbit skeletal muscle actin, Cys-374, is crosslinked, with high efficiency, to the amino group of Lys-191 in an adjacent molecule. The two reactive maleimide groups in PDM are 1.2–1.4 nm apart. These results show that Cys-374 and Lys-191 are both near an actin–actin interface and that the SH group of Cys-374 of one actin monomer is 1.2–1.4 nm from the amino group of Lys-191 of an adjacent actin monomer. The exact amino acid residues involved in an intermolecular crosslink have been identified, and we propose that this information, in conjunction with information about the location of Lys-191 and Cys-374 within the three-dimensional structure of the actin monomer, will be helpful in determining exactly how the actin monomers are associated in F-actin.

METHODS

Actin was isolated, as described earlier (7), from an acetone powder of the hind leg and back muscles of domestic white rabbits. All operations were carried out at 0–4°C. The acetone powder was extracted with G-actin buffer (2 mM Tris chloride/0.2 mM $CaCl_2$/0.2 mM ATP/0.1 mM dithiothreitol, pH 8.0), clarified by low-speed centrifugation, and polymerized by addition of 2 M KCl to a concentration of 0.1 M. After 30 min of polymerization, the F-actin was pelleted by centrifugation at 50,000 rpm in a Beckman Ti 60 rotor for 45 min, and the supernatant was decanted. The pellets were suspended in G-actin buffer and homogenized in a glass homogenizer. The protein concentration was adjusted to about 3 mg/ml, and the solution was dialyzed overnight against 30 vol of G-actin buffer. It then was centrifuged at high speed as described above, and the concentration was determined (A_{290} of 0.56 = 1 mg/ml). In preparation for crosslinking, the G-actin was polymerized and brought to pH 9.0 by dialysis overnight against 30 vol of 2.5 mM disodium tetraborate/0.1 M KCl/1 mM $MgCl_2$/0.2 mM ATP, pH 9.0 (6). [^{14}C]PDM (^{14}C in the maleimide moiety) was then added, as described by Knight and Offer (6), at the rate of 1.0 mol per mol of actin. The reaction proceeded for 30 min at 22°C and was terminated by addition of 2-mercaptoethanol. Urea was added to a concentration of 8 M, and nonradioactive *N*-ethylmaleimide was then added (100 mol/mol of -SH groups) and allowed to react 60 min; the lysine amino groups were then blocked by addition of succinic anhydride (8). The crosslinked, *S*-alkylated, succinylated actin was then dialyzed against water, lyophilized, dissolved in a $NaDodSO_4$-containing buffer, and chromatographed on a 1.9 × 200 cm column of Sephacryl S-200 (9, 10). The peak that contained crosslinked actin (see *Results*) was pooled, dialyzed against 0.1% 2-mercaptoethanol, and lyophilized. Residual $NaDodSO_4$ was removed by dissolving the powder to a concen-

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Abbreviation: PDM, *N,N'-p*-phenylenedimaleimide.

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tration of 2.0 mg/ml in 6 M urea/0.05 mM Tris acetate, pH 7.8, containing 0.01 M mercaptoethanol and passing this solution over a 0.9 × 3 cm column of "Extracti-Gel" (Pierce). The protein was detected, recoveries were estimated by measurements of A_{280} and radioactivity, and the protein-containing effluent was dialyzed against 0.5% $(NH_4)HCO_3$, pH 8.5/0.1% butanol. The protein was then digested with trypsin (5% by weight) for 16 hr at 20–25°C. The digest was lyophilized, redissolved in the digestion buffer to a concentration of 5–10 mg/ml, and chromatographed on a 1.9 × 400 cm column (two 200-cm columns in tandem) of Sephadex G-50–80 equilibrated and run in the $(NH_4)HCO_3$ buffer described above. Fractions (5 ml) were collected, A_{280} was measured, and radioactive peaks were located by liquid-scintillation counting of 50 μl from each tube. The radioactive peaks were pooled, reduced in volume, and analyzed by high-performance liquid chromatography (HPLC) as described below. Amino acid and sequencer (Beckman 890C) analyses were carried out as described (10).

Polyacrylamide gel electrophoresis was carried out using Sepragel (Separation Sciences, Attleboro, MA) gradient (10–20%) gels.

RESULTS

The crosslinking of F-actin by PDM, as measured by the appearance of dimers, trimers, and higher oligomers, followed a time course similar to that described by Knight and Offer (6). Since the monomers that remained after crosslinking could not contain intermolecular crosslinks, they were separated from the dimers and higher oligomers by gel filtration on S-200 (Fig. 1). The peak that contained dimers and higher oligomers was pooled and digested with trypsin after the removal of $NaDodSO_4$. Digestion was restricted to arginyl bonds because the ε-amino groups of lysine had been succinylated. The digest was chromatographed on Sephadex G-50–80 (Fig. 2), and the radioactivity was mainly confined to two regions, one where peptides 15–20 residues long were expected, and the other where very small peptides emerge.

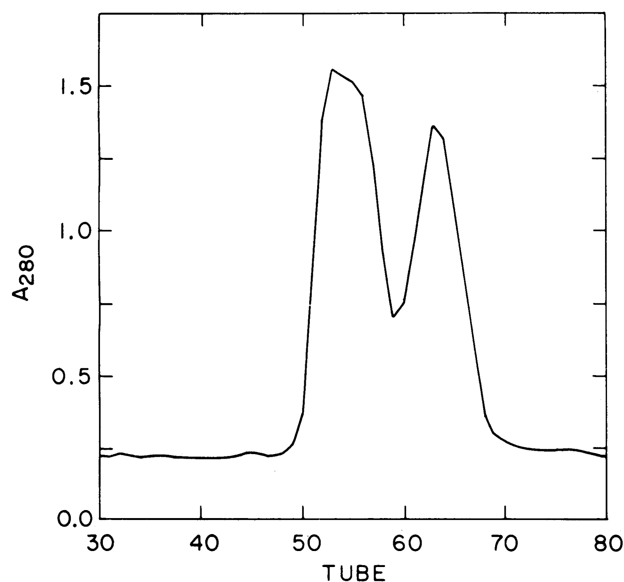

FIG. 1. Chromatography of PDM-crosslinked F-actin. A Sephacryl S-200 column (1.9 × 200 cm) was equilibrated with 50 mM Tris·HCl, pH 7.5/1% $NaDodSO_4$/1 mM EDTA (9). Denatured, S-alkylated actin was applied (70 mg in 10 ml), and 5-ml fractions were collected at 25°C at a flow rate of 22 ml/hr. Tubes 50–57 contained crosslinked dimers and higher oligomers; this peak was pooled and used for isolation of the crosslinked peptide. The second peak (tubes 60–67) contained monomers and was discarded.

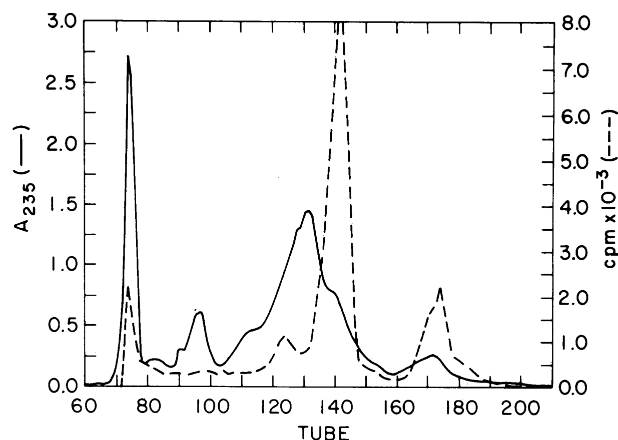

FIG. 2. Chromatography of an arginine-specific tryptic digest of crosslinked actin. Fractions (5 ml) were collected at a flow rate of 15 ml/hr and at 22°C. The major radioactive peak collected comprised tubes 135–147, and the late minor peak, tubes 166–178.

About 10% was found at the void volume; since no succinylated tryptic peptides emerge here, it was assumed to be incompletely digested material.

The major radioactive fraction (tubes 135–147 of Fig. 2) was pooled, and the peptides were separated by HPLC (Fig. 3). The only radioactive peak was subjected to amino acid analysis (Table 1). Its composition coincided precisely with the sum of two arginyl peptides, residues 184–196 and residues 373–375; cysteine was eluted before aspartic acid, as S-

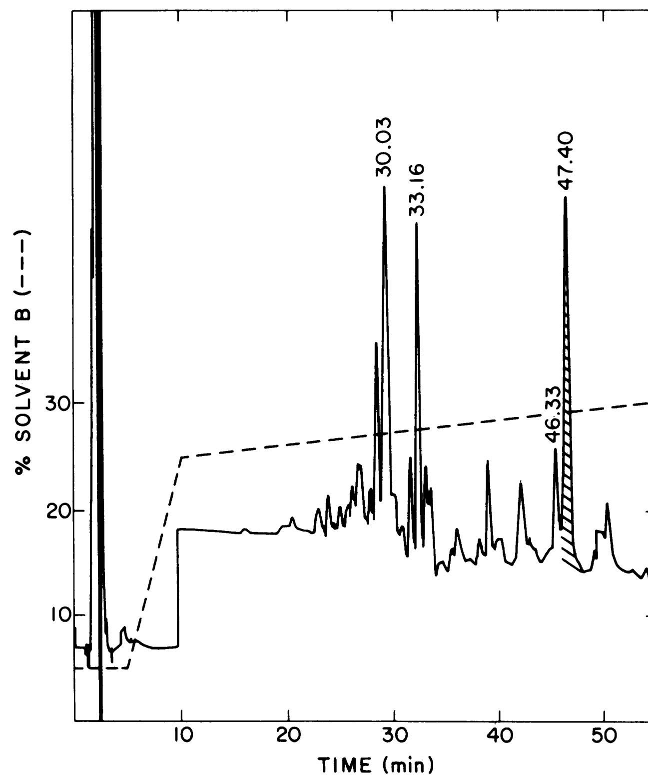

FIG. 3. HPLC of the major radioactive peak from Fig. 2. About 10 nmol of the peptide mixture in 50 μl of 50% formic acid was applied. The instrument was a Hewlett-Packard 1084B chromatograph; the column, a Hibar EC RP-18 (10-μm pore size; 4 × 250 mm). The column was run at 30°C at a flow rate of 2.0 ml/min, and detection (solid line) was at 215 nm at a setting of 0.2 absorbance units full scale. Solvent A was 10 mM ammonium acetate (pH 6); solvent B was acetonitrile. The crosshatched peak contained the radioactive crosslinked peptide.

Table 1. Amino acid composition of the radioactive crosslinked peptide

Amino acid	Composition
1,2-Dicarboxyethyl-cysteine	0.90 (1)
Aspartic acid	1.98 (2)
Threonine	1.79 (2)
Serine	0.31 (0)
Glutamic acid	1.24 (1)
1,2-Dicarboxyethyl-lysine	0.96 (1)
Glycine	0.38 (0)
Methionine	0.71 (1)
Isoleucine	1.09 (1)
Leucine	3.01 (3)
Tyrosine	0.87 (1)
Phenylalanine	0.99 (1)
Lysine	1.03 (1)
Arginine	1.00 (1)

Numbers in parentheses reflect the compositions calculated from the amino acid sequence of actin.

(1,2-dicarboxyethyl)cysteine (the hydrolysis product of a maleimide-cysteine adduct), while one of the lysyl residues appeared as *N*-(1,2-dicarboxyethyl)lysine (the hydrolysis product of a maleimide-lysine adduct), and was eluted immediately after proline (6). The yield of this peptide was about 70%.

The other radioactive peak obtained from the G-50 column (tubes 168–178 of Fig. 2) was also pooled and partially resolved by HPLC. The compositions of the peptides isolated from this fraction indicated that the radioactivity was associated with the tripeptide 373–375, in which the cysteine had been converted to the maleimide derivative. This peptide was present in about 20% yield.

The amino acid sequence of the larger radioactive peptide was determined on the automatic sequencer, and the results (Table 2) indicate that the peptide has the structure depicted in Fig. 4. Since the protein was treated with succinic anhydride before digestion, all of the lysine residues unprotected by PDM were succinylated, and the appearance of phenylthiohydantoin-*N*-succinyl-lysine at step 1 indicated that this lysine was not involved in the crosslink. The radioactivity observed at step 2 probably resulted from partial acid hydrolysis of bonds on either side of the phenylene moiety; the PTH derivatives of several possible modified cysteines would be released, all of which would possess a radioactive maleimide group. A majority of the radioactivity was found in step 8, which corresponds to Lys-191. These results prove that the crosslinking of F-actin with PDM takes place between the SH group of Cys-374 and the ε-NH- group of Lys-191 of adjacent molecules.

Table 2. Sequencer analysis of 36 nmol of the crosslinked peptide

Cycle	dpm	Amino acid	Amount of PTH, nmol
1	0.0	Aspartic acid	4.1
		N-succinyl-lysine	7.5
2	92	Leucine	12.0
3	19.7	Threonine	3.5
		Phenylalanine	1.0
4	5.7	Aspartic acid	3.3
5	0.0	Tyrosine	7.1
6	2.0	Leucine	6.7
7	5.6	Methionine	4.6
8	135	*N*-(1,2-dicarboxyethyl)lysine	1.4
9	19.8	Isoleucine	1.4
10	9.2	Leucine	3.4
11	—	Threonine	0.7
12	—	Glutamic acid	2.9

Phenylthiohydantoin (PTH) derivatives of amino acids were identified and quantified by HPLC. The radioactivity observed at cycle 2 was assumed to arise from PTH-*S*-(1,2-dicarboxyethyl)cysteine that was released by partial hydrolysis within the crosslink. "dpm" represents the disintegrations measured in aliquots taken from each tube obtained from the sequencer before conversion and HPLC analysis.

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185                 190                 195
Asp-Leu-Thr-Asp-Tyr-Leu-Met-Lys-Ile-Leu-Thr-Glu-Arg
                            |
                            | 375
                        Lys-Cys-Phe
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FIG. 4. The sequences of the fragments that constitute the crosslinked peptide. The crosslinker is represented by the vertical line between Lys-191 and Cys-374. In F-actin, the lower sequence would be contributed by one actin monomer and the upper sequence by an adjacent actin.

DISCUSSION

The results presented here demonstrate that the major, and perhaps the only, crosslink formed by reaction of PDM with F-actin is between Lys-191 and Cys-374. The fact that dimers and higher oligomers are formed indicates that intermolecular crosslinking takes place. The argument that this is an intermolecular crosslink also can be based upon the yield of crosslinked peptide obtained from actin into which 1 mol of PDM was incorporated per mol of actin and from which monomers had been removed. Crosslinked peptides were isolated from dimers and higher oligomers only, and these would contain $N - 1$ intermolecular crosslinks per oligomer, where N equals the number of actin units in the oligomer. Based upon gel patterns, it was estimated that the average size of the oligomers was four; therefore, the theoretical yield of crosslink peptide would be 0.75 per mol of actin. The observed yield was ≈70%, thus providing additional evidence for the intermolecular origin of the peptide; furthermore, only one type of crosslink was found, indicating that both the first and second reactions were highly specific for particular residues. About 20% of the radioactivity in the digested oligomer was associated with the sulfhydryl group of the uncrosslinked tripeptide Lys-Cys-Phe. This product would result from the failure of the second phase of the PDM crosslinking to react with an amino acid side chain. It presumably hydrolyzes, terminating the polymerization.

Cys-374 was previously shown to be the first -SH group of actin to react with *N*-ethylmaleimide (11) and, therefore, was expected to be the site of initial reaction of PDM. However, Lys-191 has not been attributed any special properties or reactivities. Studies on the reactivities of lysyl residues (12, 13) have suggested that Lys-191 is accessible to solvent but is rather unreactive and that its reactivity does not change detectably when F-actin is depolymerized. Its ε-amino group is presumably at the surface of actin, and its reactivity in the second phase of the crosslinking results from the proximity of the maleimide and ε-amino group after one maleimide of PDM reacts with Cys-374. It is not clear whether the PDM is "locked down" in some particular orientation on the surface of actin or whether it rotates freely but reacts with Lys-191 because it is the only lysyl residue located at the correct distance from Cys-374 for reaction. Inspection of the amino acid sequence of actin (14) reveals that there are no lysines near Lys-191: on the amino-terminal side, the closest one is Lys-113; on the COOH-terminal side, the nearest is Lys-213. A lysine residue immediately precedes Cys-374, but PDM is presumably too long to react with the adjacent side chain, and the next lysyl in the sequence is Lys-359.

The crosslinking reaction is done at pH 9.0 in order to facilitate the reaction of the maleimide group with the lysyl amino group. We cannot be sure that the high pH does not alter the structure of F-actin, but ATPase measurements showed that neither the pH 9 incubation nor the introduction of the crosslink inhibits the ability of actin to activate the ATPase of myosin S-1; therefore, we conclude that crosslinking and the conditions accompanying it do not perturb the structure of actin.

The principal conclusion to be drawn from these results is that, in F-actin, the -SH group of Cys-374 of one actin unit is located exactly 1.2–1.4 nm from the $-NH_2$ group of Lys-191 of an adjacent actin unit. Since this appears to be the first time that the exact residues involved in an intermolecular crosslink have been identified, there is no reference system that can be used to verify the validity of this type of distance measurement. However, Hartman and Wold (15) identified two intramolecular crosslinks in ribonuclease, and their assignments of interresidue distances agree with the distances deduced from the crystal structure of ribonuclease (16). Therefore, the 1.2- to 1.4-nm distance between Cys-374 and Lys-191 is probably correct.

The potential usefulness of these results cannot be assessed at this time, but they are likely to be helpful in orienting actin monomers within F-actin. When the three-dimensional structure of the actin monomer is solved and various potential contact points for intermolecular interactions are evaluated, it will be necessary to consider only those that permit Cys-374 and Lys-191 to be 1.2–1.4 nm apart; this restriction should substantially limit the number of possible orientations. Whether the packing can then be predicted with a high degree of confidence is not clear, since there are some key gaps in our knowledge of the organization of F-actin. For example, it is not known whether actin is a two-start helix, in which each actin interacts with four (or perhaps three) neighbors, or a one-start helix, with two interactions per monomer (1). For purposes of discussion, both types of helix can be viewed as two strands of subunits coiled about each other, and intermolecular crosslinking therefore could involve monomers in the same strand or, alternatively, could involve monomers that lie diagonally across from one another in the different strands. Both Knight and Offer (6) and Mockrin and Korn (17) have discussed how one might distinguish between these possibilities, but we feel that neither our data nor the earlier studies permit one to decide which type of crosslink is formed. In either case, the crosslink information presented here should be helpful for orientation.

Sutoh (18) has recently shown that *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), a bifunctional reagent that spans about 0.8 nm, crosslinks Cys-374 and one of the three lysine residues in the CNBr peptide, CB-17. CB-17 contains Lys-191, Lys-213, and Lys-215, and, in view of the results presented here, it seems likely that MBS crosslinks the same residues as does PDM. If an additional crosslink, involving two different residues, can be found, there would probably be only one way to orient the molecules to satisfy the distance requirements of two crosslinks.

Studies analogous to these may also be helpful in establishing the actin–myosin interaction sites. It has been shown that actin and myosin S-1 can be chemically crosslinked (19, 20); identification of the exact residues involved may, in the context of the crystal structures of actin and myosin S-1, lead to an understanding of which parts of these proteins constitute the actin–myosin interface.

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