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Cross-Linking Study on Skeletal Muscle Actin: Assignment of the Lysyl Residue Intermolecularly Cross-linked with Cys 373 on the Neighboring Molecule in F-Actin*

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F-actin was intermolecularly cross-linked with *N,N'-p*-phenylenedimaleimide. The cross-link was introduced between Cys-373 of an actin molecule and one of lysines of the neighboring actin molecule closely associated with their contact sites. Tryptic hydrolysis and cyanogen bromide-cleavage of cross-linked actin revealed fifteen lysines (among nineteen of intact actin) were free and not the sites of the cross-link, leaving Lys 118, Lys 213, Lys 215, and Lys 358 as candidates of the cross-linking site.

In connection with the tryptic digestion of cross-linked actin, susceptibility of 2-(N^{ε}-lysyl)succinic acid half (ethyl or phenyl) amide to trypsin was investigated with RNase A as a model system. The N^{ε}-modified lysyl peptide bonds were resistant to trypsin.

KEY WORDS: *N,N'-p*-phenylenedimaleimide/ Tryptic Hydrolysis/ Tryptic Peptides of Actin/ Susceptibility of N^{ε}-alkyllysine to Trypsin/ Structure of F-actin/ N^{ε}-alkyllysine-RNase A/

I. INTRODUCTION

Once a protein was crystallized, the structure could be solved by X-ray crystallography at the finest level establishing the atomic coordinates of constituting atoms. Most of globular proteins have such possibility if they stay monomeric or even if they form oligomeric clusters which could be accommodated into a unit lattice of three-dimensional crystals. But this is not the case for proteins in the state of linear polymers, since it is difficult to obtain cystals of such a polymer packed with a coherent lateral alignment. Actually we have no examples of analysis of linearly polymerized protein except paracrystals, which are inadequate for crystallography, although they are still valuable for low-resolution studies such as electron microscopic observations.

Actin is a protein which appears in a variety of contractile systems. It is found in most forms of life, from bacterias to vertebrates,[1] and is expected in any of motile systems in living organisms, including plants.[2] Although actin is such an important protein, under the physiological conditions, globular molecules of actin (G-actin) are assembled into a two-stranded helical polymer which is called as F-actin and the form asscciated with biological function. G-actin has been crystallized as a complex

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with DNase I* and several attempts to determine the crystal structure of G-actin have been reported,[3,4] and it is quite certain we will have the three-dimensional structure of G-actin in near future. On the contrary, it is unfortunate that such possibility will not be anticipated for F-actin, to which the biological function of actin to interact with myosin is directly associated, since good crystals adequate for crystal analysis will not be expected due to its polymeric nature.

Even if a detailed analysis of F-actin itself is impossible, to build up an approximate structure of F-actin is still possible if the three-dimensional structure of G-actin and the style of packing of actin molecule are revealed, under an assumption that the conformation of the polypeptide chain of actin molecule suffers minimal change during the change of state from G to F. This assumption is supported on the view of spectroscopic aspects which suggested no appreciable changes in the secondary structure of G- and F-actin.[5] Once we built up the structure of F-actin, the surface of an actin fiber, where the crucial interactions between actin and other fundamental proteins, myosin and tropomyosin, would take place, could be investigated and these interactions would be understood with their atomic details. So far as the tertiary structure of the unit globular actin is approximated from the crystal structure of G-actin, how to derive the packing mode of a unit molecule? There will be several ways to establish the arrangement of a unit molecule in a helical array, the most direct method is to find out the contact area between unit molecules in F-actin. In the present approach which will be described here, we introduced a chemical cross-link of definite length between polypeptide chains of neighboring subunit and attempted to analyze the position of the cross-link in the terms of the amino acid sequence of actin. Once determined the position of the cross-link, information of the distance between the residues on a neighboring pair of actin molecules will establish a mutual arrangement of the molecule, the structure of F-actin.

II. EXPERIMENTAL

Cross-linked actin. Actin was extracted from dried rabbit skeletal muscle and purified according to Spudich and Watt.[6] Actin was cross-linked with *N,N'-p*-phenylenedimaleimide as reported by Knight and Offer,[7] then the remaining cysteinyl residues were pyridylethylated with 4-vinylpyridine in 6M guanidinium chloride. About 100 mg of cross-linked and pyridylethylated actin was fractionated on a column of Sephacryl S-300 (superfine, Pharmacia), 2.2×140 cm, equilibrated and eluted with 6M urea, pH 4 (adjusted with formic acid). A pooled fraction I in Fig. 1 was dialyzed against 0.1 M acetic acid, lyophilized, termed as cross-linked actin hereafter, and used for further work. The monomer, oligomer, and polymer compositions of Fraction I to III were estimated by a densitometry of the polyacrylamide gel stained with Coomassie Brilliant Blue R-250 after SDS-gel electrophoresis.

Tryptic digestion. Fraction F-1-I of Fig. 1 (50 mg) was digested with 1 mg of trypsin

* Abbreviations used: CB, cyanogen bromide-cleaved peptide; DNase I, bovine pancreatic deoxyribonuclease I; HPLC, high performance liquid chromatography; RNase A, bovine pancreatic ribonuclease A; SDS, sodium dodecylsulfate; TLC, thin layer chromatography.

(Worthington, grade TRTPCK) in 5 ml of 0.1 M ammonium bicarbonate at 37°C for 4 hr. The precipitate was separated from the supernatant by centrifuge (about 2,000 rpm), washed twice with 0.1 M ammonium bicarbonate. The washings were combined with the supernatant, and the solution (1/50 was removed for analysis of lysine) was lyophilized.

Determination of lysine after tryptic digestion. 1/50 of the trypitc digests (see above) was deproteinized with phosphosalicylic acid and subjected to a short column analysis on an amino acid analyzer. The yield of lysine was 2.4 mol per mol of actin unit.

Ion exchange chromatography of tryptic peptides. The lyophilized tryptic peptides which were soluble in 0.1 M ammonium bicarbonate were dissolved in 0.15 M pyridine-acetic acid (pH 3.1) and chromatographed on BioRad AG50W X-2, 0.9×110 cm. The linear gradient elution was carried out at 40°C with 500 ml of 0.2 M pyridine-acetic acid (pH 3.1) and 500 ml of 2 M pyridine-acetic acid (pH 5.0), finally to 5 M pyridine-acetic acid (pH 6.0). Ninhydrin analysis after alkaline hydrolysis was applied to yield the chromatogram (Fig. 2). Dimethylsulfoxide was used instead of methylcellosolve to make a ninhydrin solution. Fractions corresponding to each peak were pooled and lyophilized.

Analysis of tryptic fractions. Aliquots from fractions F-2-1, F-2-2, F-2-3, and F-2-4 were directly acid hydrolyzed and analyzed for their amino acid composition because two-dimensional peptide mapping showed that these fractions were single component (F-2-1 and -2) or contained only minor contaminants (F-2-3 and -4). Further fractions were analyzed after purification by peptide mapping, which was carried out on a 20×20 cm silica gel plate (thickness of silica gel, 0.2 mm, Merck or Funagel from Funakoshi). The first dimensional chromatography was achieved with chloroform: methanol: conc. aq. ammonia=2:2:1,[8] the second-dimensional electrophoresis was carried out after wetting the plate with pyridine:acetic acid water=2:20:978 (pH 3.5) or pyridine : acetic acid : water=100 : 3 : 897 (pH 6.5), at 800–1,000 V for 50–60 min under a constant wattage mode. A home-built apparatus was used for TLC-electrophoresis throughout the work. The plate was not immersed in liquid coolant such as kerosene, but directly cooled by placing on an aluminum block which was circulated with cold water (about 5°C). To minimize the evaporation of buffer from the plate, the plate was covered with a glass plate separated by a spacer of 1 mm thickness. After mapping had been finished, the dried plate was stained with fluorescamine (Roche) to develope spots, which were scraped off and extracted with 25% aq. acetic acid and analyzed for their amino acid compositions after total acid hydrolysis (Fig. 3A-E). Sometimes, for example, F-2-6, it was necessary to stain the plate with potassium iodide-starch after chlorination to detect peptides whose amino terminal was blocked (acetylated or pyroglutamyl peptides). The yield of the peptides was calculated from their amino acid analysis.

Gel filtration and analysis of tryptic peptides of cross-linked actin. 60 mg of cross-linked actin was digested with 1.2 mg of trypsin in 5 ml of 0.1 M ammonium bicarbonate at 37°C. The digestion mixture was lyophilized, then dissolved in 60% acetic acid and chromatographed on Sephadex G-50 (2×150 cm) which was equilibrated and eluted with the same solvent. Aliquot of each fraction was analyzed by ninhydrin

reaction after alkaline hydrolysis. A pooled fraction F-4-I (Fig. 4) was concentrated *in vacuo* and diluted with water. After pH of the solution was adjusted to 6.5 with pyridine, the precipitate (F-4-I, insoluble) was separated by centrifugation. The supernatant, F-4-I soluble, was electrophoretically fractionated into three parts: nearly neutral or immobile, cathode-migrating, and anode-migrating fractions. The electrophoresis was carried out on a silical gel plate at pH 6.5. The nearly neutral peptides fraction was further fractionated by two-dimensional peptide mapping (electrophoresis at pH 3.5) in the manner as described as above (Fig. 3F).

Cleavage of cross-linked actin with cyanogen bromide. Cross-linked actin (22.4 mg) was dissolved in the mixture of guanidinium chloride (3 g), 2-mercaptoethanol (2 ml), and water (3 ml). The solution was adjusted to pH 8 with ammonium bicarbonate, kept at 37°C for 24 hr, dialyzed against aq. ammonium bicarbonate. The lyophilized material (20 mg) was dissolved in 1 ml of 70% aq. formic acid and treated with 100 mg of cyanogen bromide at 25°C for 24 hr. The solvent and the reagent were removed to afford the residue which was dissolved in small amount of formic acid–60% aq. acetic acid (1:1) and applied on a column of Sephadex G-50 (fine, 1.0×135 cm) equilibrated and eluted with 25% formic acid–25% acetic acid in water. The eluate was monitored at 280 nm and with ninhydrin reaction after alkaline hydrolysis. Fraction F-5-II (Fig. 5) was chromatographed on phosphocellulose as described by Elzinga.[9] The yield of peptide CB-8 (residues 283–298) was 65%.

Modification of RNase A with N-ethyl- or N-phenylmaleimide. 25 mg of RNase A (purified by IRC-50 (Mallinckrodt, CG-50) chromatography) was dissolved in 2 ml of 8M guanidinium chloride, 0.2 M Tris-HCl, pH 8.0, and reduced with 0.1 ml of 2-mercaptoethanol at 37°C for 3 hr under nitrogen. Carboxymethylation was achieved by adding 0.3 g of sodium iodoacetate to the solution (pH was readjusted to 8.0 with sodium hydroxide solution) and by keeping the mixture at 37°C for 1 hr. The solution was dialyzed and lyophilized to give *S*-carboxymethyl-RNase A (22 mg), which was dissolved in 2 ml of 0.1 M *N*-ethylmorpholine (pH 8.0): 1 ml was used for the reaction with *N*-ethylmaleimide and the remaining 1 ml for the reaction with *N*-phenylmaleimide. 35 mg of *N*-phenylmaleimide in 1 ml of dimethylformamide or 25 mg of *N*-ethylmaleimide in 1 ml of water was added to each solution and pH of these solution was adjusted to 8.0 by adding a drop of *N*-ethylmorpholine. After the reaction at room temperature for 24 hr, these solutions were dialyzed at alkaline pH, divided into several tubes, and digested with trypsin.

Tryptic digestion of the modified RNase A. Each of the modified RNase A was treated with 1/50 (w/w) amount of trypsin (Worthington, grade TRTPCK) for various time up to 4hr. Reduced and *S*-carboxymethylated RNsae A was also digested with trypsin in the similar manner.

Edman degradation of the modified, reduced and S-carboxymethylated RNase A. Edman degradation was carried out manually according to the usual way.[10] Identification and quantitation of amino acid phenylthiohydantoins were achieved by HPLC (JASCO TriRotar II equipped with Zorbax ODS (DuPont)) by the procedure of Zimmerman *et al.*[11] α-Aminoisobutyric acid was included in the Edman reaction and used as an internal standard of the analysis in the respect of both the yield of the reaction

and the position of amino acid phenylthiohydantoins on HPLC.

Amino acid analysis. Acid hydrolysis was carried out with 6N (constant-boiling) hydrochloric acid at 110°C for specified hours in a sealed and evacuated tube. A JASCO amino acid analyzer with a ninhydrin system was used to analyze the hydrolyzates. Regular pH 3.25 buffer was modified slightly acidic to separate N^{ε}-succinyllysine from proline. In our single column system (Shodex HC 095 resin), arginine, tryptophan, and *S*-(2-pyridyl)ethylcysteine appeared at 86, 95, and 100 min, respectively.

RESULTS

Cross-linked polymer of actin. Rabbit skeletal muscle actin was cross-linked with *N,N'-p*-phenylenedimaleimide (Knight and Offer[6]). Analysis of the products with SDS-polyacrylamide gel electrophoresis showed the intermolecularly corss-linked molecular species distributing among a wide range of molecular weight as reported previously.[6,12] Because both ends of the cross-linked polymer have no further intermolecular cross-links, determination of the position of intermolecular cross-links should be easier if we worked with as possible as higher polymers. Therefore the cross-linked products were fractionated according to molecular size and the fractions containing higher oligomers, fraction F-1-I in Fig. 1, were used for the further work. The distribution of monomer, dimer, and higher oligomers in each fractions, derived from SDS-polyacrylamide gel electrophoresis, are shown in Table I, showing that the amount of monomeric and dimeric actin in fraction F-1-I was less than a quarter of the total amount. Since thiols are most reactive to maleimide among the functional groups in protein and since Cys 373* is the only crysteine which is capable of a reaction

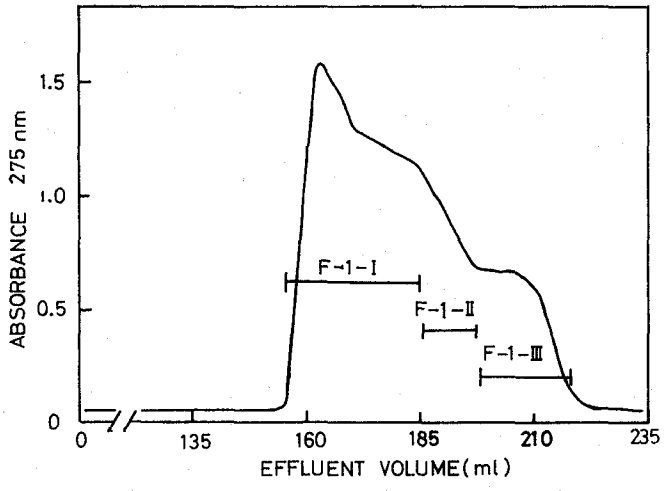

Fig. 1. Fractionation of F-actin cross-linked with *N,N'-p*-phenylenedimaleimide on Sephacryl S-300. Elution was carried out with 6 M urea, and the effluent was monitored at 275 nm. Three fractions, F-1-I to -III, were pooled.

* The numbering system appeared in Ref. 14 is conserved in the present paper. The extra serine found in the later work[15] is thus refered as Ser 234A and the total number of amino acids in actin is 375.

Table I. Yield and composition of fractions F-1-I to -III.

	Fractions		
	F-1-I	F-1-II	F-1-III
monomer	8.3%	7.1%	69.8%
dimer	16.6	53.9	21.0
trimer	19.1	25.7	4.6
tetramer	17.1	9.0	1.7
pentamer	38.9	4.3	2.9
Yield, mg (from 100 mg of mixture)	50	15	20

Table II. Amino acid composition of rabbit skeletal muscle actin, cross-linked actin, and tryptic digests.

	F-1-I[6]	actin[5]	tryptic soluble[6,7]	tryptic insoluble[6,8]
Suc-C[1]	0.73		0.33	0.23
Asp	32.6	34	18.4	13.5
Thr	24.0	27	12.4	14.3
Ser	18.3	22	8.8	14.8
Glu	41.2	39	22.5	23.8
Pro	21	19	9.5	9.0
Suc-K[2]	0.61		0.43	0.33
Gly	28.0	28	15.6	15.7
Ala	31.3	29	16.6	18.9
Val	20.5	21	9.7	13.1
Met	15.7	16	7.5	11.4
Ile	29.0	30	13.1	17.4
Leu	25.3	26	13.2	13.2
Tyr	15.9	16	7.5	12.7
Phe	12.0	12	6.4	10.3
Lys	17.6	19	11.8	7.0
His	8.4	8	4.8	5.1
3-Me-His[3]	1.2	1	1.0	0.0
Arg	18.0	18	10.1	7.9
PEC[4]	3.6		2.7	1.8
Cys	0.0	5	0.0	0.0

1) *S*-succinylcysteine. 2) N^{ε}-succinyllysine.
3) 3-methylhistidine. 4) *S*-(2-pyridyl)ethylcysteine.
5) Calculated from the sequence. 6) Hydrolyzed at 110°C for 75 hr.
7) Normalized to 3-Me-His=1.0. 8) Normalized to Lys=7.0.

with maleimides (for example, see Ref. 13), one end of the cross-link must be Cys 373, the other end is most probably one of lysines in the neighboring actin unit. Such a cross-link affords *S*-succinylcysteine and N^{ε}-succinyllysine after acid hydrolysis, and actually the ratio close to 1:1:1 (molar ratio of these two amino acids to mol of actin) was found in the acid hydrolyzate of cross-linked actin (Table II). The ratio also suggested that the number of cross-link was one per mol of actin unit.

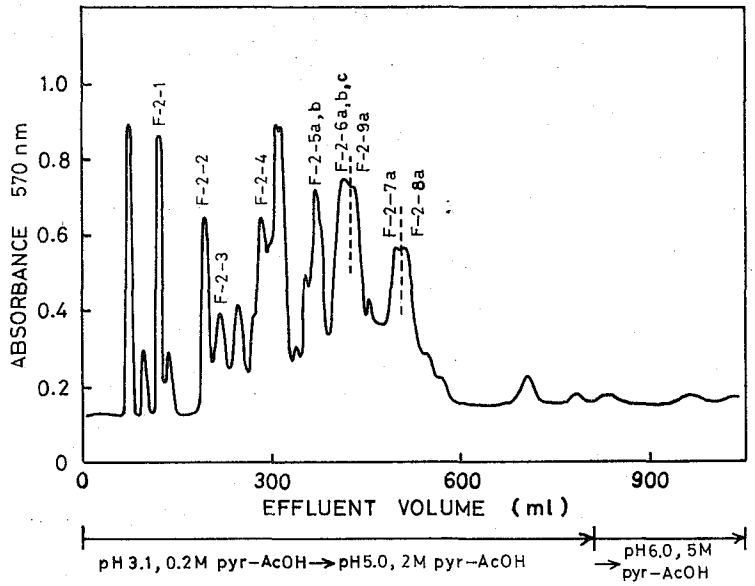

Fig. 2. Dowex 50 (BioRad AG50W X-2) chromatography of pH 8-soluble tryptic peptides of cross-linked actin. Gradient elution from pH 3.1, 0.2 M to pH 5.0, 2 M, and finally to pH 6.0, 5 M pyridine (pH adjusted with acetic acid). The ordinate represents ninhydrin reaction after alkaline hydrolysis.

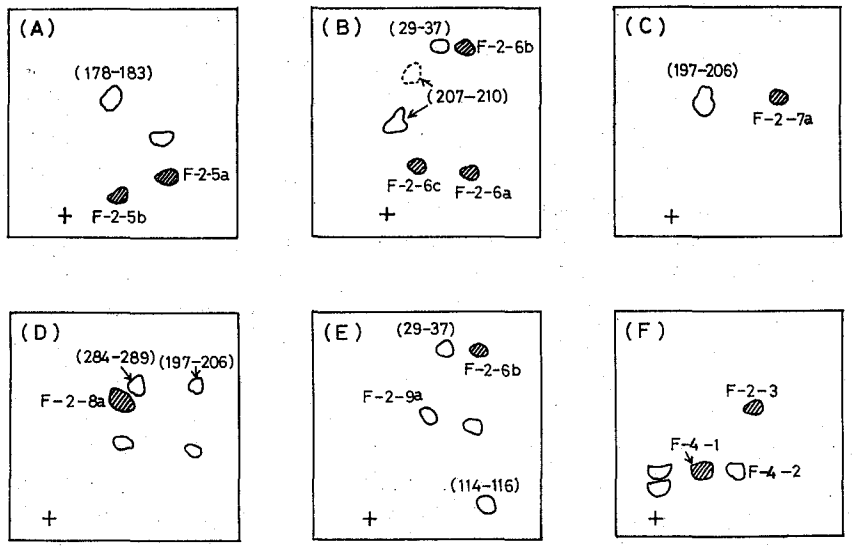

Fig. 3. Two-dimensional separation of tryptic peptides soluble at pH 8. These peptide maps were taken on silica gel TLC plates (20×20 cm). Mapping was started by ascending chromatography with $CHCl_3$: CH_3OH: conc. ammonia=2: 2: 1, followed by high-voltage electrophoresis at the specified value of pH. Shaded and unshaded areas mean lysyl and arginyl peptides, respectively. +marks represents the location where the sample was applied. (A) Fraction F-2-5. Electrophoresis at pH 6.5. (B) Fraction F-2-6, at pH 6.5. (C) Fraction F-2-7, at pH 6.5. (D) Fraction F-2-8, at pH 3.5. (E) Fraction F-2-9, at pH 6.5. (F) A neutral part of Fraction F-4-I, pH at 3.5. Anode is right side.

Table IIIA. Amino acid analysis of soluble tryptic peptides having lysine as N-terminal.[1]

	F-2-1 (1–18)[2]		F-2-2 (51–61)		F2-3 (315–325)		F-2-4 (96–113)		F-2-5a	F 2-5b	F-2-6a	(117–118)[4]
Asp	3.00	4	1.75	2	1.11	0	1.38	1				
Thr	1.71	2			1.83	2	1.69	2				
Ser	0.98	1	1.46	2	1.07	1						
Glu	2.20	2	1.95	2	1.19	1	2.11	3	1.04	0.96	1.00	1
Pro					1.27	1	3.80	4				
Gly	2.41	2	1.40	1	1.21	0	0.90	0	0.41			0
Ala	1.45	1	1.00	1	1.77	2	1.33	2				
Val	2.00	2	0.94	1	0.56	0	0.85	1				
Met					1.06	1						
Ile	1.95	2			1.02	1	0.58	0				
Leu					1.29	1	2.04	3				
Tyr			1.05	1	0.66	0	0.56	0				
Phe												
Lys	1.15	1	0.89	1	0.85	1	1.08	1	1.00	1.03	0.90	1
His							1.00	1				
3-Me-His												
Arg												
PEC[3]	0.68	1										
Yield (%)	78		73		70		67		8	19	5	

1) Amino acid contents less than 0.4 were omitted. 2) ( ): Assigned residue number.
3) *S*-(2-pyridyl)ethylcysteine. 4) Or, (214–215).

Tryptic peptides of cross-linked actin. Ion exchange chromatography. Tryptic digests of cross-linked actin was separated into a soluble and an insoluble part at pH 8. The soluble part was chromatographed on Dowex 50 (Fig. 2), fractions corresponding to chromatographic peaks were analyzed for their purity by peptide mapping which was performed with a combination of chromatography and electrophoresis on a silica gel TLC plate. Some of the fractions were nearly pure for their component (F-2-1 to -4), and the other fractions composed of peptide mixtures were further separated by peptide mapping (Fig. 3), and all these purified peptides were acid hydrolyzed and analyzed for amino acid compositions. The results, including the sequences which were assigned, for all of the lysyl peptides identified are shown in Table IIIA, and some of the arginyl peptides relevant to the present study in IIIB. The other arginyl peptides are omitted because they may not have much meaning. Although F-2-5a and -b gave the same composition after acid hydrolysis, the electrophoretic mobility suggested that less mobile F-2-5b might be a glutamyl peptide and more mobile F-2-5a might be the corresponding glutaminyl peptide. In skeletal muscle actin, however, no Gln-Lys sequence has been reported, and the present suggestion of the presence of Gln-Lys sequence might be due to a transamidation during protein manipulation or likely to an inhomogeneity of the actin amino acid sequence. F-2-6a was also considered as Gln-Lys. The combined yield of F-2-5a, -b, and F-2-6a was slightly higher than other peptides but perhaps yet insufficient to conclude the presence of 2 mol of the sequence Glu-Lys in cross-linked actin. Tryptic digestion

	F-2-6b (63–68)		F-2-6c (312–314)		F-2-7a (326–327)		F-2-8a (40–50)		F-4-1 (69–84)	
Asp									3.00	3
Thr	0.84	1							1.00	1
Ser									0.60	1
Glu			0.91	1			2.12	2	2.98	2
Pro										1
Gly	1.08	1					3.12	3	1.54	1
Ala										
Val							1.84	2		
Met			0.61	1			1.18	2	1.03	1
Ile	0.88	1			0.90	1			2.12	3
Leu	2.29	2								
Tyr									0.83	1
Phe										
Lys	1.15	1	1.00	1	1.00	1	1.00	1	1.28	1
His							0.71	1		
3-Me-His									0.78	1
Arg										
PEC[3)]										
Yield (%)	31		25		27		22		15	

Table IIIB. Peptides having arginine as C-terminal.

	F-2-9a (192–196)		F-4-2 (238–253)	
Asp			2.16	2
Thr	0.61	1	1.03	1
Ser			1.06	1
Glu	0.92	1	2.91	3
Pro			1.10	1
Gly			2.40	2
Ala				
Val			0.69	1
Met				
Ile	0.67	1	1.16	2
Leu	0.82	1	1.03	1
Tyr			0.91	1
Phe				
Lys				
His				
Arg	1.00	1	1.00	1
Yield (%)	28		20	

also afforded 2.4 mol of free lysine, which was obtained by direct amino acid analysis of the digest.

Both of the tryptic peptides, soluble and insoluble at pH 8, contained cross-linked lysine as will be seen in Table II. The ratio of N^{ε}-succinyllysine in the acid hydrolyzates was almost 1:1, when normalized to the sum of lysine and arginine, for tryptic peptides soluble and insoluble at pH 8. Therefore, cross-linked peptide(s) must be present somewhere in the chromatographic fractions of the soluble peptides, however, amino acid analysis showed that all the fractions contained cross-linked lysine (practically, it means N^{ε}-succinyllysine after acid hydrolysis) utmost 10% of the value of lysine or arginine. Much effort has been paid to isolate the cross-linked peptide(s) from the fraction which were relatively rich in succinyllysine, however, no definite evidence of cross-linked peptide has not been obtained at the present stage. One reason why a peptide fraction containing the required amount of succinyllysine was lost might be due to deposition of such a peptide owing to its high hydrophobicity or poor solubility during chromatography.

For the peptides insoluble in aq. ammonium bicarbonate, digestion with chymotrypsin or thermolysin was attempted. But the large amount of insoluble materials still persisted to remain, perhaps due to resistance of insoluble peptides to enzymatic digestion, and no clear conclusion was obtained.

Tryptic peptides of cross-linked actin. Gel filtration. In this procedure, the whole tryptic digest was dissolved in 60% acetic acid and fractionated on Sephadex G-50 (Fig. 4). Fraction F-4-I was separated into soluble and insoluble parts at pH 6.5 and the soluble part was further fractionated by electrophoresis and peptide mapping. Electrophoresis at pH 6.5 gave a nearly neutral, or of low mobility, fraction, which afforded peptides F-4-1 and F-4-2 upon peptide mapping (Fig. 3F). Amino acid analysis of these peptides showed that F-4-1 corresponded to the sequence 69–85 and F-4-2, which was an arginyl peptide (see Table IIIB), to 238–253.

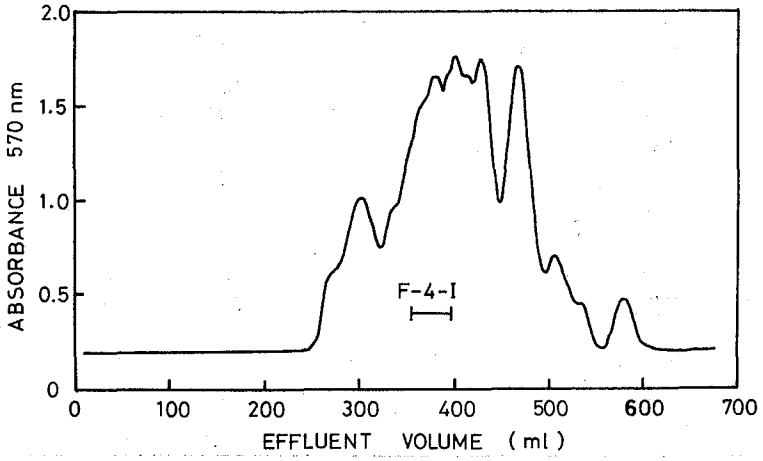

Fig. 4. Sephadex G-50 gel chromatography of tryptic peptides of cross-linked actin. Elution was carried out with 60% acetic acid and the effluents were analyzed by ninhydrin reaction after alkaline hydrolysis.

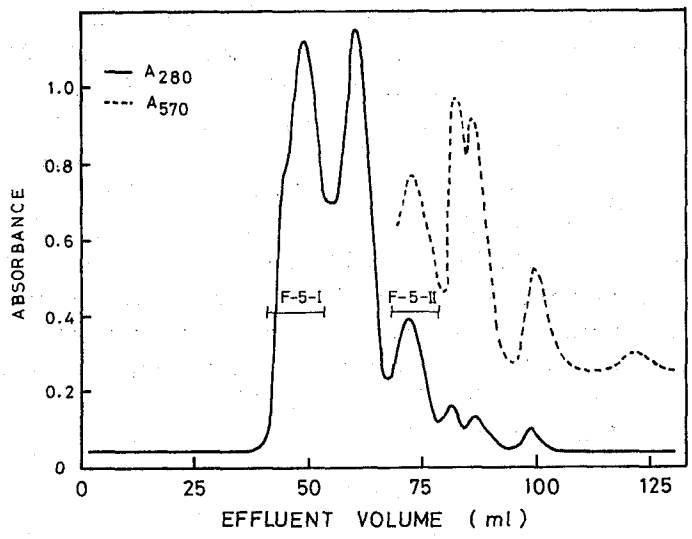

Fig. 5. Sephadex G-50 gel chromatography of cyanogen bromide peptides of cross-linked actin. The effluents were monitored by absorption at 280 nm (solid line) and ninhydrin reaction after alkaline hydrolysis (broken line).

Degradation of cross-linked actin with cyanogen bromide. To minimize the amount of oxidized methionine, cross-linked actin was treated with 2-mercaptoethanol[16] prior to the cleavage reaction, then reacted with cyanogen bromide to cleave the methionyl peptide bonds. The product was fractionated by a gel filtration on Sephadex G-50 (Fig. 5). Amino acid analysis showed only the leading peak F-5-I contained N^{ε}-succinyllysine after acid hydrolysis. Fraction F-5-I was fractionated by solubility at pH 6.5, and N^{ε}-succinyllysine was found largely in the insoluble part. Further attempts to isolate the cross-linked peptides by using enzymatic digestion (chymotrypsin was used) have not yet yielded a success.

The comparison of the chromatogram shown in Fig. 5 with the Sephadex G-50 chromatogram for cyanogen bromide peptides of intact actin (not shown) revealed that the peak F-5-II was only 1/4 for its height to the corresponding peak observed in the latter chromatogram. Phosphocellulose chromatography was used to separate the peptides in F-5-II as previously reported[9]. Only CB-8 (terminology of cyanogen bromide peptides due to Elzinga *et al.*[14]) was found in F-5-II with the yield of 65%. Among CB-peptides of intact actin, CB-9 was reported to appear also at the position corresponding to F-5-II. Much reduction in the peak height of F-5-II will be explained by the absence of peptide CB-9 which contained one tryptophan and one tryosine, compared to one tyrosine in CB-8.

Tryptic hydrolysis of lysine-modified RNase A. Reduced and *S*-carboxymethylated RNase A was modified with *N*-ethylmaleimide or *N*-phenylmaleimide at pH 8. The degree of modification was revealed by amino acid analysis after acid hydrolysis, which showed 1.5 and 1.2 mol of lysine per mol of *N*-ethylmaleimide- and *N*-phenylmaleimide-modified protein, repectively. These values indicated approximately nine lysyl

residues among ten included in RNase A were N^{ε}-alkylated with these reagents. A part of histidine was also modified as the analysis gave only 1.0 to 2.2 mol of this amino acid per mol of modified proteins instead of 4 mol for the protein without maleimide-modification as expected from the amino acid sequence. These lysine-modified RNase A and the parent protein were digested with trypsin and extent of the hydrolysis of the lysyl peptide bonds was investigated by Edman degradation of the digests. Among amino acids following the lysyl residues, aspartic acid (reduced yield, 15% for both of maleimide-modified RNase A. The reduced yield refers to the aspartic acid phenylthiohydantoin obtained from Edman analysis of tryptic digests of reduced and *S*-carboxymethylated RNase A) and tyrosine (reduced yield, 13% and 21% for *N*-ethyl- and *N*-phenylmaleimide-modified protein, respectively) were observed after 4 hr digestion. The yield of phenylthiohydantoin of phenylalanine was less than 1% of N^{ε}-unmodified protein.

DISCUSSION

The reason why we selected the cross-linker, *N,N'-p*-phenylenedimaleimide, should be mentioned at first. (1) The reagent is a rigid molecule, having reactive double bonds separated approximately 9.5 A. Due to rigidity of the cross-link, the distance between the cross-linked sites falls in a narrow range of 11–12 A. This is a quite different property distinguished from that of flexible cross-linkers, with which a precise distance may not be evaluated. (2) The reaction has been shown to produce actin oligomers or polymers in good yield and the first reactive site is no other than Cys 373. (3) As Cys 373 is the only site readily reactive to maleimides and the other four cysteinyl residues are not reactive in the absence of denaturants, the intermolecular cross-link introduced in F-actin by the reaction of *N,N'-p*-phenylenedimaleimide is between Cys 373 in one of actin unit and ε-amino group of one of lysyl residues in the neighboring actin unit. The acid hydrolysis cleaves and destroys the cross-link itself but the lysine and cysteine included in the cross-link are still recovered as the uniquely modified amino acids, N^{ε}-succinyllysine and *S*-succinylcysteine, and can be distinguished from other intact lysine or cysteine on amino acid analysis. Amide type cross-linkers, which are most frequently used in modification of lysines, regenerate lysine by acid hydrolysis and lysines incorporated in cross-links cannot be discriminated from unmodified lysines. The position of N^{ε}-succinyllysine on amino acid analysis, which had been claimed to overlap with that of proline,[17] is sensitive to pH of the eluting buffer, and N^{ε}-succinyllysine could be placed just between Pro and Gly when pH of a buffer was slightly lowered than the normal 3.25. Once established the analytical method for *N,N'-p*-phenylenedimaleimide-cross-link, our strategy to locate an intermolecular cross-link on the amino acid sequence of actin was to isolate and determine the amino acid sequence of cross-linked peptide(s) among peptide fragments produced by enzymatic or chemical cleavage of cross-linked actin. Although it has not yet been possible to get positively the cross-linked peptide, fifteen lysyl residues among nineteen included in an actin molecule were found not the site of an intermolecular cross-link. Therefore, it is possible to conclude that the cross-linking site (just peripheral to the mutual contact site of actin

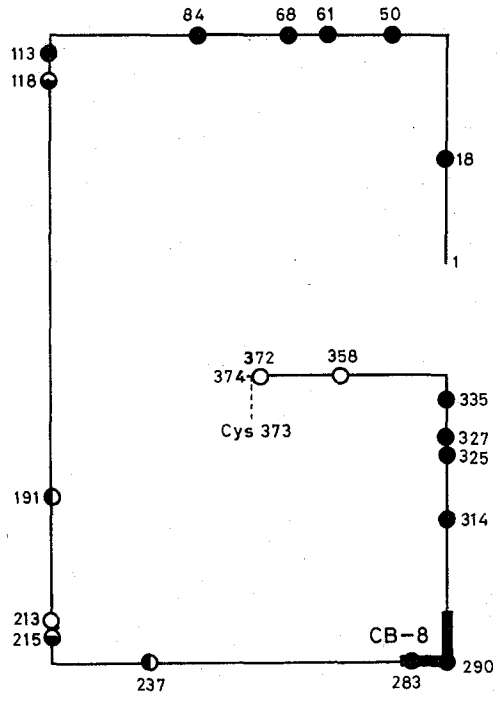

Fig. 6. The position of lysyl rsidues on the amino acid sequence of rabbit skeletal muscle actin. Filled circles, ●, mean lysyl residues being free after cross-linking, and at least either of lower-half filled circles, ◒, is free, too. It is suggested that the residues 191 and 237, ◐, are also not the site of cross-linking because the peptides following these residues are identified. An intermolecular cross-link with *N,N'-p*-phenylenedimaleimide is between Cys 373 on one actni molecule and one of lysines represented by circles, ○, on another.

molecules in F-actin) is one of the remaining four lysyl residuse, Lys 118, Lys 213, Lys 215, and Lys 358.

The lysyl groups whose N^{ε}-groups are free, namely the lysyl residues which are not incorporated into a cross-link, are summarized in Fig. 6. Lysines at 18, 50, 61, 68, 84, 113, 314, 325, and 327 were recovered after tryptic digestion of cross-linked actin, and it was conlcuded from the yield of these peptides and the composition of oligomers of cross-linked actin (Table I) that these lysines were not the site of cross-link. The result that more than 2 mol of free lysine was found in the tryptic digest of cross-linked actin suggests that Lys 290 and Lys 335 are also free from a cross-link. Since intact actin will afford 3 mol of lysine on digestion with trypsin, the missing lysine was assigned to Lys 372, which was close to Cys 373 and had a possibility to be incorporated into a cross-link in the manner to give an intramolecular cross-link between Cys 373 and Lys 372. Lys 283 (and Lys 290) was excluded from the reason that the peptide CB-8 (residue 283–298) was obtained in good yield after the cleavage with cyanogen bromide. A peptide having a sequence of Glu-Lys was obtained in slightly higher yield than that of the other peptides, so at least one of Lys 118 and 215 was free because it was less likely that both of these two ly-

sines were evenly modified. There remains a possibility that both of Lys 118 and 215 are free, but it is uncertain at moment. Lysyl peptides terminated with Lys 191 and Lys 237 have not yet been identified, but the peptides, following Lys 191 and Lys 237, 192–196 and 238–253 (both are arginyl peptides) have been isolated in reasonable yield. Since susceptibility of N^{ε}-alkyllysyl peptides to tryptic hydrolysis is much reduced less than 1/10 of N^{ε}-unsubstituted lysyl peptides as will be mentioned later, isolation of these arginyl peptides would provide an evidence that Lys 191 and Lys 237 were not the sites of substitution at their ε-amino groups. It is quite inconceivable that Lys 372, the closest neighbor of the Cys 373, is the site for intermolecular cross-link if we consider the polymerization of actin is in the manner of head-to-tail, longitudinally to the fiber of F-actin. Mockrin and Korn also showed the *N,N'-p*-phenylenedimaleimide-cross-linked actin dimer had the polarity of F-actin.[12]

These assignments of free lysyl residues leave Lys (118, 213), 215, and Lys 358 as candidates of the site of cross-linking (from the reason described above, one of Lys 118 and Lys 213 is free). There are some evidences about the lysyl groups whose ε-amino groups become unreactive or in other sense, buried, on polymerization of G-actin into F-actin. Hitchcock-De Gregori *et al.*[18] studied the structure of actin by measuring the relative reactivities of lysines with acetic anhydride using a competitive labeling technique comparing monomeric G-actin and filamentous F-actin. According to their results, lysines 50, 61, 68, 113, and 290 became unreactive during polymerization. The similar result was obtained by Lu and Szilagyi by a surface labeling method, limited reductive methylation.[19] Lys 283 was also included in these unreactive lysines in F-actin in the latter study. In the present study, a cross-link between Cys 373 on one actin molecule and lysine on another was introduced in the polymeirzed or F-state of actin. Therefore, the cross-linked lysine might not be one of these buried lysines, but on the periphery of the molecular contact site. Very recently, Sutoh reported the very similar work to the present study, using *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester as an intermolecular cross-linker for F-actin.[20] From SDS-gel electrophoretic comparison of intact and cross-linked actin, he concluded that peptide CB-17 (Lys 191, 213, 215) was the site of the cross-link, the result well consistent with the present one.

An actin molecule should have two kinds of intermolecular contact sites, lateral and longitudinal to the axis along a F-actin fiber. If the molecular symmetry of actin molecules in a molecular assembly was uniform (not alternating), the existence of polymers higher than dimer suggests the present cross-links interjoined actin molecules longitudinally to the F-actin fiber axis.

There have been many controversies upon the susceptibility of N^{ε}-alkyl- or -aryllysines to trypsin. Therefore one important question, whether trypsin can digest a peptide bond of N^{ε}-modified lysine or not, is associated with the present study, which deals with a peptide contains a derivative of succinyllysine. Even in the simplest case, N^{ε}-methyllysine, Martinez *et al.*[21] claimed the nearly same rate of tryptic hydrolysis at N^{ε}-methyllysine as at the unmodified lysines in flagellin, a protein composing bacterial flagellars, on the other hand, Joys and Kim[22] reported

much reduced susceptibility in the similar digestion of flagellin. N^{α}-Benzoyl-N^{ε}-methyllysyl amide and N^{ε}-methyllysine ethylester were hydrolyzed by trypsin at the relative rate of 1/13 of the corresponding arginylamide and 1/17 of lysine ethylester, respectively.[23] N^{ε}-dimethyl- and N^{ε}-trimethyllysine have been reported not the substrate of trypsin.[24-26] N^{ε}-carboxymethyllysine was claimed not to be hydrolyzed with trypsin[27] but no data were available for succinyllysine or its derivatives among literatures. So we took a tryptic digestion of RNase A, which was intact or fully modified with *N*-ethylmaleimide or *N*-phenylmaleimide at lysines, as a model system.

According to N-terminal analysis of the peptide mixtures obtained by tryptic hydrolysis of these N^{ε}-modified protein, some of amino acids (aspartic acid and tyrosine) following to the lysine in the original amino acid sequence were obtained, but in reduced yield in comparison to the N^{ε}-unmodified RNase A. It means that peptide bonds Lys* 37–Asp 38 (an asterisk accompanying Lys means that the residue is modified with maleimide derivatives) and Lys* 91–Tyr 92 were not completely stable to tryptic hydrolysis, nevertheless the rates of the hydrolysis were much retarded to less than 10–20% of these for lysyl peptides in which N^{ε}-amino groups were free. The peptide bond between Lys* 7 and Phe 8 was completely stable to trypsin under the conditions used. Other peptide bonds, Lys* 31–Ser 32, Lys* 98–Thr 99, and Lys* 104–His 105 were more or less stable to trypsin, but a quantitative aspect has not yet been achieved due to ambiguities inevitable to Edman analysis of serine and threonine and modification at histidine. It is possible that the susceptibility of N^{ε}-alkyllysyl peptide bonds to trypsin is sequence-specific and perhaps more extended works will be necessary to establish the problem.

X-Ray crystallographic works on actin have been in progress for G-actin–DNase I complexes[3,4] but availability of F-actin crystals is hopeless. The construction of the structure of F-actin, the species which interacts with myosin to act as a mechanochemical system in living organisms, therefore, critically requires a knowledge of the packing mode of G-actin into F-actin. The present result will affford a restriction on the scheme of association of actin molecule and will provide a basis for construction of F-actin structure.

REFERENCES

(1) A. Weeds, *Nature*, **296**, 811 (1982).
(2) T.C. Pesacreta, W.W. Carley, W.W. Webb, and M.V. Parthasarathy, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 2898 (1982).
(3) D. Suck, W. Kabash, and H.G. Mannherz, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 4319 (1981).
(4) N. Sakabe, K. Sakabe, K. Sasaki, H. Kondo, T. Ema, N. Kamiya, and M. Matsushima, *J. Biochem.*, **93**, 299 (1983).
(5) E.W. Taylor, *Ann. Rev. Biochem.*, **41**, 577 (1972).
(6) J.A. Spudich and S. Watt, *J. Biol. Chem.*, **246**, 4866 (1971).
(7) P. Knight and G. Offer, *Biochem. J.*, **175**, 1023 (1978).
(8) R.E. Stephens, *Anal. Biochem.*, **84**, 116 (1978).
(9) M. Elzinga, *Biochemistry*, **9**, 1365 (1970).
(10) T. Koide in "Seikagaku Zikken Koza", The Japanese Biochemical Society Ed., Vol. 1 (II), Tokyo Kagaku Dojin, Tokyo, 1977, pp. 158–177.
(11) C.L. Zimmerman, E. Appela, and J.T. Pisano, *Anal. Biochem.*, **77**, 569 (1977).

(12) S.C. Mockrin and E.D. Korn, *J. Biol. Chem.*, **256**, 8228 (1981).
(13) P. Detmers, A. Weber, M. Elzinga, and R.E. Stephens, *J. Biol. Chem.*, **256**, 99 (1981).
(14) M. Elzinga, J.H. Collins, W.M. Kuehl, and R.S. Adelstein, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2687 (1973).
(15) R.C. Lu and M. Elzinga, *Biochemistry*, **16**, 5801 (1977).
(16) R.S. Adelstein and W.M. Kuehl, *Biochemistry*, **9**, 1355 (1970).
(17) C.F. Brewer and J.P. Riehm, *Anal. Biochem.*, **18**, 248 (1967).
(18) S.E. Hitchcock-De Gregori, S. Mandala, and G.A. Sachs, *J. Biol. Chem.*, **257**, 12573 (1982).
(19) R.C. Lu and L. Szilagyi, *Biochemistry*, **20**, 5914 (1981).
(20) K. Sutoh, *Biochemistry*, **23**, 1942 (1984).
(21) R.J. Martinez, J.H. Shaper, N.P. Lundh, P.D. Bernard, and A.N. Glazer, *J. Bacteriol.*, **109**, 1239 (1972).
(22) T.M. Joys and H. Kim, *Biochim. Biophys. Acta*, **581**, 360 (1979).
(23) L. Benoiton and J. Deneault, *Biochim. Biophys. Acta*, **113**, 613 (1966).
(24) M. Gorecki and Y. Shalitin, *Biochem. Biophys. Res. Commun.*, **29**, 189 (1967).
(25) L. Poncz and D.G. Dearborn, *J. Biol. Chem.*, **258**, 1844 (1983).
(26) J.H. Seely and N.L. Benoiton, *Can. J. Biochem.*, **48**, 1122 (1970).
(27) S. Korman and H.T. Clarke, *J. Biol. Chem.*, **221**, 133 (1956).