Gln-41 is intermolecularly cross-linked to Lys-113 in F-actin by *N*-(4-azidobenzoyl)-putrescine

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Abstract

The bifunctional reagent *N*-(4-azidobenzoyl)-putrescine was synthesized and covalently bound to rabbit skeletal muscle actin. The incorporation was mediated by guinea pig liver transglutaminase under conditions similar to those described by Takashi (1988, *Biochemistry* 27, 938–943); up to 0.5 M/M were incorporated into G-actin, whereas F-actin was refractory to incorporation. Peptide fractionation showed that at least 90% of the label was bound to Gln-41. The labeled G-actin was polymerized, and irradiation of the F-actin led to covalent intermolecular cross-linking. A cross-linked peptide complex was isolated from a tryptic digest of the cross-linked actin in which digestion was limited to arginine; sequence analysis as well as mass spectrometry indicated that the linked peptides contained residues 40–62 and residues 96–116, and that the actual cross-link was between Gln-41 and Lys-113. Thus the γ -carboxyl group of Gln-41 must be within 10.7 Å of the side chain (probably the amino group) of Lys-113 in an adjacent actin monomer. In the atomic model for F-actin proposed by Holmes et al. (1990, *Nature* 347, 44–49), the α -carbons of these residues in adjacent monomers along the two-start helices are sufficiently close to permit cross-linking of their side chains, and, pending atomic resolution of the side chains, the results presented here seem to support the proposed model.

Keywords: actin; cross-link; mass spectrometry; photoaffinity label; sequence

Actin is both a cytoskeletal and a myofibrillar protein that appears to be ubiquitous in eukaryotes. Both the primary (Elzinga & Collins, 1975) and tertiary (Kabsch et al., 1990) structures of actin from rabbit skeletal muscle are known, and an atomic model for F-actin has recently been proposed (Holmes et al., 1990). A rigorous test for this model is chemical cross-linking, wherein a bifunctional reagent is used to cross-link an amino acid side chain in one actin monomer with a reactive side chain in an adjacent monomer within the polymeric F-actin. Because the length of the cross-linker can be calculated, the vectorial displacement of the groups that react with opposite ends of the bifunctional reagent can be estimated. We previously demonstrated that *N,N'*-phenylene-dimaleimide (Knight & Offer, 1978) cross-links the sulfhydryl group of Cys-374 with the ϵ -amino group of Lys-191 (Elzinga & Phelan, 1984). In the studies reported

here we have synthesized a new bifunctional reagent, *N*-(4-azidobenzoyl)-putrescine (ABP), incorporated it into rabbit skeletal muscle actin, and showed that it cross-links Gln-41 with Lys-113. These studies are based upon the work of Gorman and Folk (1980), who first proposed and carried out the incorporation of amino-group-containing aryl azido compounds into proteins by transglutaminase (TG)-mediated coupling of the amino moiety to the carboxyl groups of accessible glutamine side chains, and the work of Takashi (1988), who showed that TG coupled the free amino group of the fluorophore dansyl-cadaverine into Gln-41 of rabbit skeletal muscle G-actin.

Results

Selection of the reagent

We designed a heterobifunctional reagent that had two attributes. First, it could be incorporated into actin by TG, and second, it carried a photoactivatable group. For

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the latter, the azido group was selected because of the availability of azido-containing compounds as the starting material for synthesis. Putrescine was selected as the diamine because it was the shortest alkyl diamine that, when coupled to the 4-azidobenzoyl moiety, could be incorporated into actin. *N*-(4-azidobenzoyl)-ethylenediamine and *N*-(4-azidobenzoyl)-diaminopropane, the two- and three-carbon homologues, were not incorporated into G-actin by TG, although these diamines can be coupled by TG to actin and to the glutamine side chain of benzoxy-carboxyl-glutaminyglycine (data not shown).

Labeling

In pilot studies of incorporation of ABP into actin, we were able to achieve substoichiometric labeling with G-actin, but essentially no labeling of F-actin. The incorporation into G-actin generally approached 0.5 asymptotically, and after 4–5 h of reaction, the actin was viscous and birefringent, suggesting that it was polymerizing. We assumed that the polymerization was induced by the 1 mM CaCl_2 in the solution, which was required by the TG for activity. Reduction of the CaCl_2 concentration reduced incorporation, presumably because it reduced TG activity. Sonication of the reaction mixture during the latter part of the reaction increased incorporation, but we were unable to achieve incorporation greater than 0.56 M/M. The reaction conditions that we settled upon gave labeling of 0.43–0.56 moles of label per mole of actin in four separate experiments.

Isolation of cross-linked oligomers

After removing excess ABP and polymerizing the labeled actin, the F-actin was cross-linked by photoactivation of the azido group. This actin was then reduced, alkylated, and succinylated, and applied to a Sephacryl S-200 HR column, 1.9×200 cm, in 0.5% NH_4HCO_3 . As shown in Figure 1, two partially resolved peaks were obtained. They were pooled, concentrated by lyophilization, and rechromatographed. The pooled peaks were analyzed by polyacrylamide gel electrophoresis; the first peak to emerge contained mostly dimers ($M_r = 86,000$) with some monomer and some higher oligomers, whereas the second peak, after rechromatography, contained >90% monomers. Both were labeled, as shown by the presence of radioactivity.

Isolation of labeled peptides

The oligomer and monomer peaks were then digested by trypsin. Because the free ϵ -amino groups of the lysines were succinylated, digestion was restricted to arginine. The digests were subjected to gel filtration on a Sephadex G-50 column, and the chromatograms are shown in Figure 2. The profiles, monitored at 220 nm to reflect to-

Fig. 1. Pattern of elution of cross-linked actin from a Sephacryl S-200 HR column. The column was 1.9×200 cm and was developed in 0.5% NH_4HCO_3 . The sample was applied to the column in 2 mL of 8 M urea. Eight-milliliter fractions were collected, the flow rate was 48 mL/h, and 10- μ L aliquots were counted.

tal peptide present, were similar and resembled those seen previously for analogous preparations (Elzinga, 1987). Both showed a sharp early peak between tubes 20 and 25, containing mixed aggregates and/or undigested material. The monomer fraction had a single symmetric labeled peak. It was pooled, lyophilized, and subjected to high performance liquid chromatography (HPLC) on an Aquapor RP-300 column in 0.1% trifluoroacetic acid (TFA), and a gradient of 10–60% acetonitrile. Several peaks were seen, of which one, emerging at 23.5% acetonitrile, was radioactive, and its amino acid composition indicated that it was not homogeneous. This fraction was rechromatographed on an IONEX A300 column in 10 mM NH_4HCO_3 , pH 7.5, eluted with a NaCl gradient; a radioactive peak that emerged at 105 mM had a composition corresponding to that expected for residues 40–62. It was sequenced, and its sequence clearly began at His-40 (Table 1). Most of the radioactivity was observed at step 2, corresponding to Gln-41. Thus, the only radioactive peak gave a single labeled peptide in which the counts were found in the position corresponding to Gln-41, indicating that the TG had incorporated ABP into Gln-41 of actin. In the monomer fraction, the radioactive ABP presented cross-linking reagent that did not react with an adjacent actin, either because it was not photoactivated or because it reacted with solvent.

When the digested oligomer peak was gel filtered (Fig. 2B) the radioactivity was distributed between two peaks. One eluted in a position corresponding to that seen with the monomer fraction. High performance liquid chromatography on Aquapor RP-300 and IONEX A300 columns (see above), followed by sequencer anal-

Fig. 2. Sephadex G-50 gel filtration of tryptic (arginine only) digests of monomeric (A) and cross-linked (B) actin. The columns were 1.9×200 cm and were developed in 0.5% NH_4HCO_3 . The flow rates were 36 mL/h, and the fraction size was 4.5 mL. Fifteen-microliter aliquots were counted. In A, tubes 37–44 (Peak A) were collected and used for isolation of the labeled, uncross-linked peptide. In B, tubes 31–37 were collected for isolation of the cross-linked peptide, and tubes 38–46 for uncross-linked peptide.

Table 1. Twelve steps of automated Edman degradation of the labeled, uncross-linked peptide^a

Step	Peptide 40–62			
	pth amino acid	Position in actin	pmol	cpm
1	His	40	46	0
2	—	41	—	525
3	Gly	42	58	210
4	Val	43	40	28
5	Met	44	38	10
6	Val	45	36	0
7	Gly	46	38	10
8	Met	47	29	0
9	Gly	48	35	0
10	Gln	49	27	0
11	Lys	50	14	0
12	Asp	51	20	0

^a About 90 pmol containing 900 cpm were applied. The absence of a pth amino acid and the presence of most of the counts, at step 2, indicated that Gln-41 was labeled by ABP.

ysis, indicated that it represented the peptide 40–62 labeled at Gln-41 and that it was not cross-linked. The peak that emerged earlier (tubes 31–37) was then studied. It was pooled as shown, lyophilized, and applied to the HPLC under the conditions used for the uncross-linked peptide. Although several peptide peaks were observed, the radioactivity was distributed as a broad, poorly resolved area, and the recovery was low. We tried other columns and gradients but were unable to find conditions under which a clear well-resolved peak was seen.

Fig. 3. Chromatography of peak A from Figure 2B on DEAE-Sephadex. The column was 0.5×1.5 cm, the flow rate was 0.5 mL/min, the fraction volume was 3 mL, and the gradient was linear, 50 mL of 0.03 M and 50 mL of 0.3 M triethylamine bicarbonate, pH 7.5. Thirty microliters/tube were counted. Tubes 10–18 were collected and applied to the column shown in Figure 4.

We then chromatographed this fraction on a column of DEAE-Sephadex developed with a gradient of 0.03–0.3 M triethylamine-bicarbonate, pH 7.5 (Fig. 3). The radioactivity emerged as a broad peak; about 50% of the counts were recovered between 0.11 and 0.175 M buffer. This fraction was collected, concentrated by lyophilization, and applied to the same G-50 column used for

Fig. 4. Sephadex G-50 gel filtration of the fraction collected from the column illustrated in Figure 3. Conditions were as described in the legend to Figure 2.

the initial peptide fractionation. In this chromatogram (Fig. 4), the radioactivity and absorption did not coincide exactly, indicating that the cross-linked peptide was still not pure. The specific radioactivity was highest in the ascending part of the curve, and we selected tubes 55, 56, and 57 for further study. Amino acid analyses were consistent with the presence of the peptide that contains Gln-41 (residues 40–62), but the composition did not permit identification of the peptide to which it was cross-linked. Aliquots from these tubes were then subjected to amino acid sequence analysis. Tube 54 gave a clear double sequence, as shown in Table 2. Inspection of the data showed that two phenylthiohydantoin (pth) amino acids were seen at most steps; one of them corresponded to that expected for the peptide 40–62, and the other for the sequence beginning at Val-96. Approximately equal amounts of the two pth amino acids were seen at each step. The recovery of counts in the sequencer steps is also shown in Table 2. The specific activity was 10 cpm/pmol, and about 1,140 cpm were applied to the sequencer.

Table 2. Sequencer data for the cross-linked complex^a

Step	Residues 40–62			Residues 96–116			cpm
	pth amino acid	pmol	Position in actin	pth amino acid	pmol	Position in actin	
1	His	41	40	Val	70	96	84
2	—	—	41	Ala	77	97	261
3	Gly	73	42	Pro	59	98	37
4	Val	73	43	Glu	73	99	12
5	Met	66	44	Glu	67	100	10
6	Val	69	45	His	28	101	0
7	Gly	56	46	Pro	39	102	0
8	Met	57	47	Thr	14	103	2
9	Gly	59	48	Leu	37	104	0
10	Gln	43	49	Leu	39	105	26
11	Lys	51	50	Thr	12	106	8
12	Asp	47	51	Glu	24	107	0
13	Ser	10	52	Ala	31	108	0
14	Tyr	30	53	Pro	20	109	0
15	Val	27	54	Leu	20	110	0
16	Gly	22	55	Asn	23	111	0
17	Asp	36	56	Pro	19	112	0
18	Glu	20	57	—	—	113	0
19	Ala	18	58	Ala	18	114	0
20	Gln	16	59	Asn	16	115	0
21	Ser	5	60	Arg	6	116	0
22	Lys	10	61				
23	Arg	9	62				

^a About 114 pmol containing about 1,140 cpm were applied. In steps 1–21, except 2, 18, and 19, a mixture of pth amino acids was observed. The only peptides with sequences that could yield the observed mixtures were 40–62 and 96–116; the amino acids were therefore placed as shown. At step 19, only pth-Ala was observed, and equal amounts were assigned to the two peptides. Gln was missing from step 2 and Lys was absent from step 18, because they were covalently coupled by APB. The lysines at steps 11 and 22 were observed as pth-succinyl-lysine.

About 400 cpm were recovered over the first five steps, 65% of which emerged in step 2.

The location of the cross-link was deduced as follows. At step two only pth-Ala was seen, indicating that the Gln-41 was modified. The expected residues were observed at all of the other steps except at step 18 where only Glu was seen. This Glu presumably represents residue 57 of actin. The other peptide, 96–116, has lysine at this position (Lys-113), and because the lysines had been succinylated, we expected to see ϵ -N-succinyl-lysine. The pth derivative of ϵ -N-succinyl-lysine eluted in our HPLC system at 10.87 min, between DMP (10.32 min) and Ala (12.45 min), and was seen at step 11 (residue Lys-50) and step 22 (residue Lys-61). Using a standard area equal to that of Ala, we computed a recovery of 51 pmol at step 11 and 10 pmol at step 22; these values are consistent with the amount expected, based upon the amounts seen in the steps before and after ϵ -N-succinyl-lysine, indicating that this derivative of lysine would be recovered in normal yields if it were present. The presence of Ala, Asn, and Arg at steps 19, 20, and 21 shows that the peptide 96–116 is intact, and thus the absence of ϵ -N-succinyl-lysine at step 18 indicates that the lysine is modified, presumably cross-linked to Gln-41.

Mass spectrometry

Additional structural information was obtained by analyzing the cross-linked peptide by mass spectrometry. An aliquot of fraction 54 from the G-50 column (Fig. 4) corresponding to about 10 pmol of peptide was loaded onto a microcapillary HPLC column and then eluted with an acetic acid/acetonitrile gradient directly into the elec-

troscopy ionization source. Shown in Figure 5 is the result obtained by summing 38 spectra recorded over a 1-min period as the sample entered the mass spectrometer. The major component in the mixture adds 4, 5, 6, and 7 protons and afford signals centered at m/z 1,298.9, 1,039.8, 866.6, and 743.0. The average mass of the $(M+H)^+$ ion calculated from these values is 5,194.3. Note that the predicted average mass of the $(M+H)^+$ corresponding to the completely succinylated peptide containing residues 40–62 cross-linked to residues 96–116 is 5,294.4. Succinylation increases the mass of a peptide by 100 Da per amino group. Accordingly, the experimentally observed mass corresponds to the cross-linked peptide minus one of the expected succinyl groups.

Electrospray ionization mass spectra were also recorded after two cycles of manual Edman degradation had been performed on the above sample. If the peptide contained residues 40–62 cross-linked to residues 96–116, the product following two cycles of Edman degradation should contain residues 98–116 cross-linked to Gln-41 as its pth derivative (see Fig. 6 for reference). If the cross-link is attached at Lys-113 and this residue is thus devoid of a succinyl group, the calculated average m/z value for $(M+H)^+$ derived from this product is 2,579.6. Under electrospray ionization conditions, the shortened peptide adds 2, 3, and 4 protons. The resulting mass spectrum contains signals at m/z 649.6, 866.3, and 1,298.5 (data not shown). The average m/z value for the $(M+H)^+$ ion calculated from these data is 2,596.1, 18 Da higher than the predicted value. Addition of water to the cross-link during the acid-catalyzed cleavage step of the Edman degradation (see Discussion) is postulated to explain this result.

Fig. 5. Electrospray ionization mass spectrum recorded on 10 pmol of cross-linked peptide as it elutes from the microcapillary HPLC column.

Fig. 6. The sequences of intermolecularly cross-linked peptides described here (above) and previously (Elzinga and Phelan, 1984) (below).

To identify the cross-linked residue in the shortened peptide, $(\text{M}+\text{H})^{+3}$ ions (m/z 866.3) from the above sample were subjected to collision-activated dissociation in the triple quadrupole mass spectrometer. In this experiment, about 10 pmol of the sample are injected into the electrospray ionization source directly from a microcapillary HPLC column. The first mass filter, quadrupole 1, is set to transmit ions within a 6-mass unit window centered at m/z 866.3. On entering the collision cell, quadrupole 2, $(\text{M}+\text{H})^{+3}$ ions suffer 10–100 low energy (15–40 eV) collisions with argon present at a pressure of 3 mtorr. During this process, ion translational energy is converted to vibrational energy, and the peptide $(\text{M}+\text{H})^{+3}$ ions undergo fragmentation, more or less randomly, at the various amide bonds along the backbone of the linear peptide chain. Elimination of small molecules such as water and ammonia and, in this case, part of the cross-linking moiety also occurs from the side chains of some amino acids. A collection of neutrals and ions carrying one and two positive charges is produced by the above processes. Neutrals are pumped away by the vacuum system, and the charged fragments are transmitted to a third mass filter, quadrupole 3, which separates them according to mass. To maximize ion transmission (sensitivity), quadrupole 3 is also usually operated at less than unit resolution.

To generate the mass spectrum in Figure 7, the mass range from 50 to 2,000 Da was scanned five times, and the resulting data were then summed. Total instrument time to acquire the data was 40 s. Fragment ions in the spectrum appear in clusters because the data system is attempting to resolve signals that are more than 1 mass unit wide and because the clusters also contain contributions from ^{13}C and other isotopes in each fragment.

Shown at the top of Figure 7 is the amino acid sequence for residues 98–116. Predicted monoisotopic masses for fragment ions of type b and type y (Hunt et al., 1986) derived from this peptide are shown above and below the sequence, respectively. Those observed in the spectrum are underlined. Fragment ions of type y all contain the C-terminus plus 1, 2, 3, etc. additional residues (Hunt et al., 1986). Subtraction of m/z values for any two fragments that differ by a single amino acid, NHCH(R)CO , and have the same number of charges generated a value that specifies the mass and thus the identity of the extra residue in the larger fragment. Ions of type y_1 – y_3 at m/z 175, 289, 360 facilitate assignment of the last three residues at the C-terminus of the peptide as Ala–Asn–Arg. The next member of the type y ion series should contain Lys, succinylated-Lys, or Lys plus the cross-link to the pth derivative of Gln-41. No signal is observed for any of these species. However, the type y_5 ion containing the

Fig. 7. Collision-activated dissociation mass spectrum recorded on the $(M+3H)^{3+}$ ions from the peptide Pro-98–Arg-116 cross-linked to Gln-41 as its pth derivative.

last five residues in the peptide is observed at m/z 1,054.5, a value that included the mass (469 Da) of the cross-link connected to the pth derivative of Gln-41. Observation of this ion suggests that the cross-link must be attached to either Pro-112 or Lys-113. Additional ions of type y present in the spectrum confirm the predicted sequence back to Glu-107.

Shown on line 2 below the sequence at the top of Figure 7 are m/z values for a series of ions of type y that are formed by loss of a 336-Da fragment from the cross-linking moiety. Signals corresponding to these ions are labeled with solid circles on the spectrum in Figure 7. Note that the mass separation between these signals allows residues 102–113 in the sequence to be specified.

Predicted fragments of type b carrying one and two positive charges are shown on the lines above the sequence in Figure 7. Those observed in the spectrum are underlined. These ions all contain the amino terminal residue plus one or more additional residues. Subtraction of m/z values for any two fragments of type b that differ by a single amino acid, $NHCH(R)CO$, generates a value that identifies the extra residue present in the larger fragment.

Assignment of Lys-113 as the cross-linked residue in the sequence stems from an analysis of the type b ions that carry two positive charges. Note that the b_{14}^{2+} ion occurs at m/z 771.9, the expected value for residues 98–111 without the cross-linking moiety. The next member of the series, b_{15}^{2+} , occurs at m/z 820.4, a shift of 48.5

Da. This increment is that expected for the addition of an unmodified Pro residue within a series of doubly charged ions. The next member of the series, b_{16}^{2+} , appears in the spectrum at m/z 1,119.1. The observed shift of 298.5 Da is that expected for the addition of a Lys residue plus the cross-linking moiety attached to the pth derivative of Gln-41. Loss of the neutral fragment having a mass of 336 Da from the singly charged b_{16} ion generates the signal at m/z 1,901.9 and provides additional support for assigning Lys-113 as the cross-linked residue within the peptide containing residues 98–116.

We conclude from the above sequencer and mass spectrometry data that ABP is bound to actin (at Gln-41) through the putrescine moiety, that upon photoactivation the cross-linker reacts with the ϵ -amino acid group of Lys-113, and that the cross-link is susceptible to hydrolysis under acidic conditions (see Discussion and Fig. 8). This instability precludes reverse-phase HPLC at low pH and explains the addition of water observed in the mass spectrum following two cycles of Edman degradation.

Discussion

Chemical cross-linking can give information about the proximities of sites to which the two reactive moieties bind, and there are basically four steps: (1) selection or synthesis of the reagent, (2) incorporation of the reagent into the biological structure by reaction of one of the functional groups, (3) generation and reaction of the second functional group, and (4) identification of the sites of reaction. In the experiments reported here, we synthesized a new reagent, ABP. Its design was based upon the work of Gorman and Folk (1980), who showed that TG could catalyze the incorporation of amines into proteins and proposed that molecules with unique properties (cross-linkers or fluorophores) could be synthesized, and of Takashi (1988), who applied this principle to actin, and showed that TG incorporated dansyl cadaverine into actin at a single site, Gln-41.

Design of the reagent used here was based upon the following considerations: (1) putrescine was the shortest alkyl diamine that, when coupled to the *N*-azido-benzoyl moiety, served as a substrate for TG; (2) the azido-group has been widely used as a photoactivatable reagent; and (3) the availability of tritiated *N*-succinimidyl-4-(azidobenzoate) enabled us to label the reagent. The reagent was incorporated into G-actin in substoichiometric amounts, but F-actin was refractory to incorporation. Because 1 mM Ca^{2+} was required for TG activity, and this level of Ca^{2+} induces polymerization, we conclude that the concurrent reactions, ABP incorporation into G-actin by TG, and polymerization of G-actin to F-actin, yielded substoichiometric incorporation of about 0.5 M/M. It may have been possible to repurify the partially modified actin, depolymerize it, and retreat it with TG and ABP to increase incorporation. However the par-

tially modified actin was suitable for resolution of the question that we posed, so further reaction was not pursued. Takashi (1988) reported incorporation of about 1 M/M of dansyl cadaverine into G-actin, using slightly different reaction conditions.

Specificity of ABP incorporation:

The putrescine moiety

Both our results and those of Takashi (1988) show that essentially all of the incorporation of the amine by TG occurs at Gln-41. Actin contains 11 glutamine residues (Elzinga and Collins, 1975); seven are in α -helices, and four are in loops or other extended conformations (Kabsch et al., 1990). Of these four, Gln-263 appears to be buried between two domains, whereas Gln-246, Gln-49, and Gln-41 are near or at the surface of G-actin (Kabsch et al., 1990). Gln-246 and Gln-49 are presumably in conformations in which their amido groups are not accessible to TG, but the published diagrams showing the locations of the α -carbons of these residues do not have sufficient detail to permit one to tell whether the side chains extend into the surrounding solvent. Because Gln-41 is accessible to TG, it seems likely that the side chain of Gln-41 extends into the solvent and that the loop of which it is a part is flexible. The fact that TG cannot incorporate reagents into F-actin suggests that Gln-41 is at or near an actin-actin interface.

Reaction of the azido group

Photolysis of an azido group yields a highly reactive product that can potentially react with any of several chemical moieties (Bayley & Staros, 1984; Hollemans et al., 1983). In the work described here, reaction was primarily, if not exclusively, with Lys-113. The evidence was as follows. Gel filtration of the digest yielded one peak, in addition to the peak that contained the uncross-linked peptide, suggesting that a single species of cross-linked peptide was formed. The sequence data obtained from the cross-linked complex (Table 2) show recovery of all the pth amino acids expected from peptides 40–62 and 96–116, except for Gln-41 (to which the putrescine is bound) and Lys-113. The mass spectrometric data (see Results) independently identified Lys-113 as the cross-linked residue.

Chemistry of the reaction

Work on model compounds has shown that photoactivation of azido-benzoyl groups yields a chemically reactive azepine, a seven-member heterocyclic ring formed by insertion of the electron-deficient nitrogen (nitrene) into the benzene ring (Fig. 8) (Li et al., 1988; Soundararajan and Platz, 1990). Reaction with a nucleophile can then occur at a carbon atom adjacent to this nitrogen, specifically

Fig. 8. Proposed reaction scheme. X represents the putrescine moiety that is part of ABP and is bound to the side chain of Gln-41; the zig-zag line and Y represent Lys-113. In reaction A photolysis of the azido-aryl moiety produces an extremely high energy electron-deficient singlet nitrene (I) that rearranges to the didehydroazepine (II). This intermediate keteneimine (rather than the initial nitrene [I]) is expected to invite nucleophilic attack by the side chain amino group of Lys-113 to yield an initial 1H-azepine (not shown), which rapidly tautomerizes to the 3H-azepine (III), the expected primary cross-linked entity. The mass spectral evidence is consistent with the formation of III. After two steps of Edman degradation an entity having the mass of the shortened peptide plus one molecule of water (18 atomic mass units) was observed. This could be IV, V, and/or Va or prototropic forms not shown. The tautomers depicted are not necessarily the most stable ones. For example breakage of the cross-link by reaction F might give rise to the amide derived from VII and VIII, which loses carbon dioxide because of the facility with which carbamic acids undergo decarboxylation. Similarly, a postulated hemiaminal intermediate (VIa), if cleaved in acid, would be expected to yield VIIa (or a tautomer) and VIIIa. These compounds were not observed, suggesting that the acidic conditions prevailing during the cleavage steps of Edman degradation were not sufficiently rigorous to effect reactions F or F'. Although these considerations do not prove the mechanism of these reactions, they do strongly suggest the exclusion of the hydrazine-mediated cross-link and offer plausible explanations for the observations made in this study.

the carbon to which the azido group was bound in the azido-benzoyl moiety. Reaction of this carbon with the ϵ -amino nitrogen of lysine would yield compound III, illustrated in Figure 8. The mass of the cross-linked peptide resulting from such a set of reactions coincides with the mass observed (Fig. 5).

It seems likely that the substituted azepine moiety in the cross-linked peptide would be susceptible to hydrolysis in acidic pH as illustrated in Figure 8, by reactions leading to the entities (intermediates and possible prototropic forms) IV, and/or V, Va. Evidence supporting this scheme was to be found in the mass spectral data after

two cycles of manual Edman degradation of the cross-linked peptide. An ion of 18 mass units greater than expected of the shortened cross-linked peptide was observed. The addition of a molecule of water (18 mass units) led to the speculation that such a hydration was consonant with the cross-link containing the azepine moiety, III. If further hydrolysis occurred past the entities V and/or Va (including tautomers not shown), to VII and/or VIIa, we should have seen ions of reduced mass, corresponding to the products of cleavage of the cross-link coupled either to peptide 40–62 or 98–116. The absence of these ions suggested that the cross-link was resistant to further

breakdown under the conditions employed during the manual Edman degradation.

When the cross-linked fraction was analyzed in the gas-phase sequencer, radioactivity was released during the first five steps, the majority emerging at step 2. A plausible explanation for this release of radioactivity would be that under the acidic conditions obtaining, tritium could be exchanging from the azepine moiety of the cross-link (Fig. 9). The tritium label in a system that is essentially a cyclic enamine is susceptible to a series of protonations and deprotonations leading to the exchange of protons/tritiums with concomitant loss of counts due to exchanges as shown. Certain tautomeric forms of the azepine system are considered more stable than others. Also, scission of the cross-link in the sequencer, by hydrolysis of VI and/or VIa, would cause release of radioactivity at step 2, because the tritium would be in the fragment bound to the Gln, VII, or VIIa (Fig. 8).

Our inability to isolate the cross-linked peptide by HPLC could be due to acid hydrolysis (reaction C or reactions C and D or D', Fig. 8) during the chromatography, which was done under acidic conditions, 0.1% TFA in H₂O and CH₃CN. If this reaction(s) occurred during the chromatography, the resolution and yield would be compromised. The fraction that was applied to the HPLC column seemed to contain intact cross-linked peptide that was stable at neutral pH, because chromatography of the sample on a DEAE column yielded the intact fragment. Ponstingl et al. (1991) have reported that peptides coupled to a photoreactive derivative of colchicine, 2-nitro-4-azidophenyl-desacetyl-colchicine were labile in the presence of HPLC separation media based on silica gel matrices, precluding HPLC on C₁₈-based columns. It is possible that our difficulties with HPLC, and perhaps automated sequencing, of this fraction were caused by the same factors.

An alternative reaction that we must consider is direct coupling of the nitrene to the ϵ -amino nitrogen of lysine. This would yield a benzoyl moiety bound to lysine

through a hydrazine ($\text{H}-\text{N}(\text{H})-\text{N}-$) linkage. The mass of such a cross-link would be identical with that of the azepine-mediated cross-link. We cannot exclude this reaction, but it seems likely that such a product would be completely stable during HPLC and Edman degradation. Because the cross-link was labile, the hydrazine-type linkage does not seem to be the major product of the photoreaction.

It is of interest to note that the azido groups of both ABP and 8-azido-ATP (Hegyi et al., 1986) reacted with lysyl side chains. In contrast to the cross-link described here, the link between 8-azido-ATP and Lys-336 was acid stable. The chemistry of the adenine ring is different from that of the benzoyl moiety, and reaction of the nitrene of photoactivated 8-azido-ATP with lysine seemed to generate a stable cross-link, probably a hydrazine. Others have reported reaction of photoactivatable azido groups with tyrosine and isoleucine side chains (Hollemaans et al., 1983), as well as with tryptophan (Okamoto and Yount, 1985), but in no case has the structure of the adduct been definitively established.

Structural implications

The intermolecular cross-link described here, as well as the one between Cys-374 and Lys-191 described earlier (Elzinga & Phelan, 1984), impose certain constraints upon models of the structure of F-actin. The tertiary structure of the monomer, G-actin, is required for interpretation because this tells us where the cross-linked residues are located on the surface of actin. The monomers must then be organized, in F-actin, such that the side chains are close enough together for the cross-links to form. The actin structures that constitute the frame of reference are the recently published G-actin structure (Kabsch et al., 1990) and F-actin model (Holmes et al., 1990). Milligan et al. (1990) have also published a model for F-actin, but its resolution is not high enough to discern individual side chains or α -carbons. For the high

Fig. 9. Tautomeric rearrangement of protons leading to tritium exchange and loss from the cross-link. The 3H-azepine form (I) is more stable than the 1H-azepine (II), which is essentially an antiaromatic and thus an unstable 8 π electron system. Furthermore, any of the entities shown in Figure 8 could lose tritium label due to acid-catalyzed protonation and deprotonation of the enamine (or potential enamine) system obtaining in III-IV-V/Va-VI/VIa.

resolution structure, the locations of the α -carbons along the polypeptide chain have been published (Kabsch et al., 1990), but not the coordinates of the amino acid side chains, limiting knowledge of side chain distances.

The Cys-374:Lys-191 cross-link

In the model of Holmes et al. (1990), the α -carbons of these two side chains are about 19 Å apart in adjacent monomers along the genetic helix; thus, this cross-link (phenylene-dimaleimide) must extend across the central axis of the polymer. In other words, if one considers F-actin to be composed of two strands, this cross-link connects Cys-374 of a monomer in one strand with Lys-191 of a monomer in the other strand. We have built models of *N,N'*-phenylene-dimaleimide and the Cys-374:Lys-191 cross-link; they reveal that the maximum distance between the two maleimide functional groups is 9.8 Å, and the maximum distance between the α -carbons is 21.4 Å.

The Gln-41:Lys-113 cross-link

Gln-41 is part of a loop that constitutes one "corner" of G-actin, in a domain that contains residues ~50 to ~70. This loop is a major contributor to the DNase I binding site of G-actin and is an important point of contact between actin monomers in the Holmes et al. (1990) model of F-actin. Lys-113 is part of an α -helix on the surface of a domain that contains the amino-terminus, the carboxyl-terminus, and residues ~75 to ~135. Gln-41 and Lys-113 are essentially on opposite sides of the G-actin sphere, 35–40 Å apart, but the α -carbons appear to be within 10–20 Å of each other in contiguous monomers along the two-start helices of the F-actin model. An atomic model of the reagent ABP shows that the maximum length of the cross-link itself is 10.7 Å, and the maximum distance between the α -carbons of Gln-41 and Lys-191 is 22.3 Å. Both cross-links can therefore be accommodated within the proposed atomic structure of F-actin (Holmes et al., 1990).

Actin-actin contact points

Lu and Szilagyi (1981) and Hitchcock-DeGregori et al. (1982) attempted to identify lysyl residues at actin-actin contact points by examining changes in reactivity of lysyl side chains that accompany polymerization. Both found that Lys-113 is much less reactive in F-actin than in G-actin, consistent with its location at an actin-actin interface. Collins et al. (1971) studied the reactivity of lysyl side chains with FDNB, and reported that Lys-113 was more reactive to this reagent in F-actin than in G-actin. They interpreted this to mean that in F-actin Lys-113 was surrounded by a hydrophobic pocket favorable for bind-

ing of FDNB, which then reacted with the ϵ -amino group of Lys-113.

Hegyi et al. (1974) found that modification (carbethoxylation) of His-40 inhibits actin polymerization; this was confirmed by Miki (1988), who reported that this reaction affects an actin-actin contact point, but not a myosin or tropomyosin-troponin binding site. His-40 is adjacent to Gln-41, and therefore the data reported here, placing Gln-41 near an actin-actin interface, corroborate conclusions drawn in the previous work. However, addition of the bulky ABP does not abolish polymerizability, indicating that Gln-41 does not participate in actin-actin interactions crucial for polymer formation. The work of Schwyter et al. (1989) has implicated residues near Gln-41 in actin-actin interactions as well as in S-1 binding. They cleaved actin at Met⁴⁷-Gly⁴⁸, and showed that the digested actin had a higher critical concentration for polymerization and a reduced affinity for myosin S-1.

In conclusion, we have described synthesis of a new photoactivated reagent that cross-links adjacent actin monomers in the two-start helix. Evidence is presented that photoactivation of the azidobenzoyl moiety results in formation of a didehydroazepine-mediated reaction with an ϵ -amino group, yielding an intermolecular cross-link that is slowly hydrolyzed in weak acid. This cross-link, as well as one described previously, can be accommodated within, and therefore supports, the F-actin model of Holmes et al. (1990). Determination of the atomic coordinates of the relevant amino acid side chains will permit refinement of the spatial localization of the cross-links.

Methods and materials

Proteins

Actin was isolated from an acetone powder of the hind leg and back muscles of domestic white rabbits. Guinea pig liver TG was isolated as described by Connellan et al. (1971).

Cross-linker

³H-N-4-(azidobenzoyl)-putrescine (Fig. 10) was synthesized as follows. *N*-hydroxysuccinimidyl-4-azidobenzoate, 10 μ mol of unlabeled (Sigma Chemical Co.) and 9.25 mBq of labeled (*N*-[benzoate-3, 5-³H], New England Nuclear) reagent, was dissolved in 100 μ L dimethylformamide and was slowly added to 100 μ mol of putrescine (Sigma Chemical Co.) dissolved in 200 μ L isopropanol, with stirring in an ice bath. The reaction mixture was incubated at 4 °C overnight. The clear supernatant, containing all of the radioactivity, was carefully separated from the white precipitate, dried under N₂, redissolved in methanol:water (1:1), and dried under vacuum. The ³H-ABP was then separated from the excess putrescine

N-(4-Azidobenzoyl)putrescine

Fig. 10. The structure of *N*-(4-azidobenzoyl)-putrescine. It is tritiated at the 3 and 5 positions of the benzene ring.

on an Alltech Maxi-Clean[™] silica cartridge prewashed with chloroform, using chloroform:methanol:acetic acid (16:4:1) for separation.

The eluted ³H-ABP was dried under N₂, redissolved in water, and dried again under vacuum to remove traces of the chromatography solution. The final product was dissolved in water and stored at -18 °C. The yield, based upon starting radioactivity, was about 60%. The product was homogeneous on silica thin-layer chromatography (TLC), gave a single radioactive ninhydrin-positive spot, and had a characteristic absorption spectrum with a maximum at 270 nm ($\epsilon_{270} = 2 \times 10^4$); the 270-nm band disappeared after photoactivation.

Labeling of G-actin with ABP by TG

G-actin at a concentration of 15 μ M (0.63 mg/mL) was incubated in a solution containing 200 μ M ABP, 400 μ M ATP, 1 mM CaCl₂, 2 mM Tris-HCl, pH 8, and 4 μ M TG at 5 °C for 5 h; during the last hour the reaction solution was subjected to several short bursts of sonication in a batch sonicator to depolymerize the actin. At the conclusion of the reaction, the actin was polymerized by addition of KCl and MgCl₂ to 100 mM and 1 mM, respectively, and the resulting F-actin was pelleted by centrifugation at 40,000 rpm in a 50 Ti rotor for 90 min. The F-actin pellet was then homogenized in 200 μ M ATP, 1 mM MgCl₂, 100 mM KCl, 2 mM Tris-HCl, pH 8, in a volume that yielded a concentration of about 1 mg/mL. Experiments were done using 20–30 mg of actin.

Photocross-linking of the labeled F-actin

The F-actin solution was placed in a pyrex beaker covered by Sealwrap, and the depth of the solution was no greater than 1 cm. The solution was gently stirred in an ice bath under N₂ for 1 h to eliminate dissolved O₂. The incorporated label was then photoactivated by irradiation of the sample by a Gelman-Camag Model 51402 Universal UV lamp at a distance of about 15 cm under a constant N₂ stream for 6 min.

Reduction, alkylation, succinylation, and separation of oligomers from monomers

Solid urea was added to the photocross-linked F-actin solution to a concentration of 8 M, and the sample was stirred for 2 h in the presence of a 400-fold molar excess of dithiothreitol; a 1,000-fold molar excess of iodoacetamide was then added and allowed to react for 30 min. The excess of iodoacetamide was quenched by adding β -mercaptoethanol in a two-fold molar excess over iodoacetamide.

The lysyl amino groups were then blocked by addition of a 500-fold molar excess of succinic anhydride over protein-bound lysine; during this reaction the pH was maintained at 7.5–8.2 by dropwise addition of 1 M NaOH. The cross-linked alkylated succinylated actin was then dialyzed exhaustively against water, lyophilized, dissolved in a minimum volume of 8 M urea, and gel filtered on a 1.9 $\times$ 200-cm column of Sephacryl S-200 HR in 0.5% (NH₄)HCO₃ (containing 0.1% *n*-butanol as a preservative), pH 8.0.

Tryptic digestion and separation of tryptic peptides

Both the monomer (uncross-linked) and oligomer (cross-linked) fractions were digested by trypsin (1% by weight; Sigma, TPCK treated) for 16 h at room temperature; after lyophilization the digests were chromatographed on a Sephadex G-50 column (1.5 $\times$ 170 cm) in 0.5% (NH₄)HCO₃ at pH 8.0.

Mass spectrometry

Mass spectra were recorded on a TSQ-70 triple quadrupole instrument (Finnigan-MAT, San Jose, California) equipped for electrospray ionization. Samples were dissolved in 0.5% (v/v) acetic acid, and aliquots were then injected into the electrospray ionization source from a fused silica, microcapillary HPLC column with an inside diameter of 75 μ m and a length of 70 cm (Hunt et al., 1991). The last 10 cm of the column was filled with C-18 packing material. Peptides were eluted with a 10-min gradient of 0–80% acetic acid (0.5%)/acetonitrile flowing at a rate of 1–2 μ L/min. Experimental conditions for recording collision-activated dissociation spectra on multiply charged ions generated in the electrospray ionization source have been described (Hunt et al., 1991).

Manual Edman degradation

Cross-linked peptide (40 pmol) was dissolved in 2 μ L of water and treated with 8 μ L of a 5% solution of phenylisothiocyanate in pyridine. The mixture was heated at 45 °C for 40 min, solvent was removed by lyophilization, and the residue was then treated with 10 μ L of anhydrous

trifluoroacetic acid for 10 min at 37 °C. The cleavage reaction was terminated by lyophilization of the reaction mixture. The residue was then dissolved in 12 μ L of water and extracted twice with 12 μ L of butyl acetate; the aqueous layer was then subjected to a second cycle of Edman degradation as described above. Extraction of the product mixture with 12 μ L of butyl acetate was performed twice. All steps were done under argon to minimize oxidation. The sample to be analyzed by electrospray ionization mass spectrometry was lyophilized, redissolved in 1 μ L of acetic acid, and then diluted to a volume of 20 μ L with water.

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References

- Bayley, H. & Staros, J.V. (1984). Photoaffinity labeling and related techniques. In *Azides and Nitrenes* (Scriven, E.F.V., Ed.), pp. 433–490. Academic Press, Inc., New York.
- Collins, J.H., Morkin, E., & Elzinga, M. (1971). Structure function studies on rabbit skeletal muscle actin. *Fed. Proc.* 30, 1630a.
- Connellan, J.M., Chung, S.I., Whetzel, H.K., Bradley, L.M., & Folk, J.E. (1971). Structural properties of guinea pig liver transglutaminase. *J. Biol. Chem.* 246, 1093–1098.
- Elzinga, M. (1987). Carboxyl group reactivity in actin. In *Methods in Protein Sequence Analysis* (Walsh, K.A., Ed.), pp. 615–623. Humana Press, Inc., Clifton, New Jersey.
- Elzinga, M. & Collins, J.H. (1975). The primary structure of actin from rabbit skeletal muscle. *J. Biol. Chem.* 250, 5897–5905.
- Elzinga, M. & Phelan, J.J. (1984). F-actin is intermolecularly crosslinked by N,N'-p-phenylene-dimaleimide through lysine-191 and cysteine-374. *Proc. Natl. Acad. Sci. USA* 81, 6599–6602.
- Gorman, J.J. & Folk, J.E. (1980). Transglutaminase amine substrates for photochemical labeling and cleavable cross-linking of proteins. *J. Biol. Chem.* 255, 1175–1180.
- Hegyi, G., Premecz, G., Sain, B., & Muhlrad, A. (1974). Selective carbethoxylation of the histidine residues of actin by diethylpyrocarbonate. *Eur. J. Biochem.* 44, 7–12.
- Hegyi, G., Szilagyi, L., & Elzinga, M. (1986). Photoaffinity labeling of the nucleotide binding site of actin. *Biochemistry* 25, 5793–5798.
- Hitchcock-De Gregori, S.E., Mandala, S., & Sachs, G.A. (1982). Changes in actin lysine reactivities during polymerization detected using a competitive labeling method. *J. Biol. Chem.* 257, 12573–12580.
- Hollemans, M., Runswick, M.J., Fearnley, I.M., & Walker, J.E. (1983). The sites of labeling of the β -subunit of bovine mitochondrial F₁-ATPase with 8-azido-ATP. *J. Biol. Chem.* 258, 9307–9313.
- Holmes, K.C., Popp, D., Gebhard, W., & Kabsch, W. (1990). Atomic model of the actin filament. *Nature* 347, 44–49.
- Hunt, D.F., Alexander, J.E., McCormack, A.L., Martino, P.A., Michel, H., Shabanowitz, J., Sherman, N., Moseley, M.A., Jorgenson, J.W., & Tomer, K.B. (1991). Mass spectrometric methods for protein and peptide sequence analysis. In *Techniques in Protein Chemistry II* (Villafranca, J.J., Ed.), pp. 441–454. Academic Press, New York.
- Hunt, D.F., Yates, J.R., III, Shabanowitz, J., Winston, S., & Hauer, C.R. (1986). Protein sequencing by tandem mass spectrometry. *Proc. Natl. Acad. Sci. USA* 83, 6233–6237.
- Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F., & Holmes, K.C. (1990). Atomic structure of the actin: DNase I complex. *Nature* 347, 37–44.
- Knight, P. & Offer, G. (1978). p-N-N'-phenylenebismaleimide, a specific cross-linking agent for F-actin. *Biochem. J.* 175, 1023–1032.
- Li, Y.-Z., Kirby, J.P., George, M.W., Poliakoff, M., & Schuster, G.B. (1988). 1,2-Didehydroazepines from the photolysis of substituted aryl azides: Analysis of their chemical and physical properties by time-resolved spectroscopic methods. *J. Am. Chem. Soc.* 110, 8092–8098.
- Lu, R.C. & Szilagyi, L. (1981). Change of reactivity of lysine residues upon actin polymerization. *Biochemistry* 20, 5914–5919.
- Miki, M. (1988). Characterization of carbethoxylated actin. *J. Biochem.* 104, 312–315.
- Milligan, R.A., Whittaker, M., & Safer, D. (1990). Molecular structure of F-actin and location of surface binding sites. *Nature* 348, 217–221.
- Okamoto, Y. & Yount, R.G. (1985). Identification of an active site peptide of skeletal myosin after photoaffinity labeling with N-(4-azido-2-nitrophenyl)-2-aminoethyl diphosphate. *Proc. Natl. Acad. Sci. USA* 82, 1575–1579.
- Ponstingl, H., Barnes, L.D., Granzow, C., Himes, R.H., Maier, G., & Nasioulas, G. (1991). Elucidating ligand binding sites in polypeptides by photoaffinity labeling with aryl azides. In *Methods in Protein Sequence Analysis* (Jörnval, H. et al., Eds.), pp. 169–176. Birkhäuser Verlag, Basel.
- Schwytter, D., Phillips, M., & Reisler, E. (1989). Subtilisin-cleaved actin: Polymerization and interaction with myosin subfragment 1. *Biochemistry* 28, 5889–5895.
- Soundararajan, N. & Platz, M.S. (1990). Descriptive photochemistry of polyfluorinated azide derivatives of methyl benzoate. *J. Org. Chem.* 55, 2034–2044.
- Takashi, R. (1988). A novel actin-label: A fluorescent probe at glutamine-41 and its consequences. *Biochemistry* 27, 938–943.