Photoaffinity Labeling of the Nucleotide Binding Site of Actin†

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ABSTRACT: Rabbit skeletal muscle actin was photoaffinity-labeled by the nucleotide analogue 8-azidoadenosine 5′-triphosphate. In both G-actin and F-actin about 25% covalent incorporation was achieved. The labeled actins were digested with cyanogen bromide, and the labeled peptides were isolated and sequenced. In F-actin the label was bound primarily to Lys-336, while in G-actin the label was bound to Lys-336 or to Trp-356. The results indicate that the nucleotide binding site is near the phalloidin binding site of actin [Vanderkerckhove, J., Deboben, A., Nassal, M., & Wieland, T. (1985) *EMBO J. 4*, 2815–2818]. The binding of the azido group to Trp-356 in G-actin but not in F-actin may indicate that a change in the conformation of actin occurs in this region.

Actin is a protein that is widely distributed in nature. It was originally identified as a muscle protein, but it is now clear that it is also a major constituent of cytoplasm in most eukaryotic cells. It seems to function in maintenance of cellular structure and in motility. Actin can exist in a globular form (G-actin), having a single polypeptide chain (375 residues in vertebrate skeletal muscle actins) of molecular weight 42 000 (Collins & Elzinga, 1975) and a noncovalently bound ATP (Straub & Feuer, 1950); see also reviews by Katz (1970) and Korn (1982). In the presence of salt, actin polymerizes to fibrous actin (F-actin), and during polymerization, the terminal phosphate is removed. The association constant for ATP–G-actin is about 7.5×10^9 M^{-1} (Huang et al., 1983), and the ATP is therefore exchangeable with nucleotides in the medium; the ADP in F-actin is more tightly bound and is essentially nonexchangeable.

The bound nucleotide is important for maintaining the native structure of actin, as indicated by the fact that actin readily denatures when the nucleotide is removed (Lehrer & Kerwar, 1972). It is not clear whether the nucleotide has an active role in other functions; one possibility is that cyclic exchange and dephosphorylation of nucleotide during treadmilling has a role in the contractile process in nonmuscle cells (Wegner, 1976; Hill & Kirschner, 1982). In an effort to identify residues that contribute to the nucleotide binding site and to determine whether changes in the binding site accompany polymerization, we have attempted to bind a nucleotide analogue to the ATP/ADP binding site of actin. Such labels have been used in several systems over the past few years, and several different nucleotide analogues have been synthesized and used for this purpose (Bayley & Knowles, 1977; Potter & Haley, 1983). Among these, photoaffinity labels having an azido group have been the most informative. They are inert in the dark, permitting study of the properties of the protein with the bound analogue, but without covalent modification of the protein. Upon illumination, a free radical is generated that reacts rapidly with neighboring residues, and because the binding is structurally specific while the covalent reaction is chemically nonspecific, photoaffinity labels are excellent tools for investigating amino acid residues in the ligand binding sites. The advantage of 8-azido-ATP over several other nucleotide derivatives is that the azido group is attached directly to the adenine ring, leading to covalent reaction of the nitrene (the free radical generated by illumination) with residues that are close to the adenine moiety of the nucleotide.

In this paper, we present evidence that 8-azido-ATP can be specifically and covalently bound to actin, that the principal site of binding is Lys-336 in both F- and G-actin, and that binding to Trp-356 also occurs in G-actin.

Experimental Procedures

Synthesis of [^{14}C]-8-Azido-ATP. The synthesis was carried out according to Schäfer et al. (1978) with some modifications. One hundred micromoles of ATP and 5–10 MBq of [^{14}C]ATP (Chemapol, Czechoslovakia) were dissolved in 2 mL of 1 M sodium acetate, pH 4.5. Four hundred micromoles of bromine dissolved in 2 mL of 1 M sodium acetate was added, and the solution was kept at room temperature for 45 min in a Teflon-capped test tube. The excess bromine was extracted by chloroform, the sample was diluted 10 times with distilled water, and the pH was raised to 8–9 by addition of 1 N NaOH. The sample was applied to a DEAE-Sephadex A25 column, and the nucleotides were eluted with a linear gradient of 0.2 and 0.6 M TEA–HCO_3, 300 mL each. 8-Bromo-ATP was obtained and was found to be homogeneous on PEI[1]–cellulose, and the absorption spectrum of the product had a maximum at 264 nm. The sample was dried in a rotary evaporator, washed 3 times with methanol, and dried over P_2O_5 for several days.

For conversion of 8-bromo-ATP to 8-azido-ATP the dried material was dissolved in 2 mL of water-free freshly distilled dimethylformamide containing a 10–20 molar excess of triethylammonium azide prepared from freshly distilled hydra-

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[1] Abbreviations: PEI, poly(ethylenimine); DEAE, diethylaminoethyl; SP, sulfopropyl; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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zoic acid. Conversion took place in the dark at 75 °C for 7 h in a tightly sealed 5-mL bottle.

The crude product (containing a mixture of mono-, di-, and triphosphates of azidoadenosine) was phosphorylated in the following way. To a 10 mM solution of the nucleotide mixture were added $MgCl_2$ and creatine phosphate to concentrations of 10 and 50 mM, respectively, and the pH was adjusted to 7.6 with Tris buffer. Phosphorylation was started by adding 20 units of creatine phosphokinase (Sigma, type III). The enzymatic phosphorylation was terminated after 25 min by adding EDTA to 5 mM.

After fractionation of the sample on DEAE-Sephadex A25 (under conditions identical with those described above), 60 μmol of 8-azido-ATP, 20 μmol of 8-azido-ADP, and about 5 μmol of 8-azido-AMP were obtained. The 8-azido-ATP fraction was lyophilized (3 times), dissolved in 50% methanol, and stored at −20 °C.

The UV spectrum of 8-azido-ATP had an absorption maximum at 282 nm, and a characteristic drop of absorbance was obtained after illumination ($\Delta_{282} = 1.1 \times 10^4$ at pH 2).

Preparation of 8-Azido-ATP–G-Actin. Actin was prepared according to Spudich and Watt (1971). After two depolymerization–polymerization cycles, the F-actin pellet was homogenized in 30 mM KCl and 2 mM Tris-HCl, pH 7.6, and ultracentrifuged again to remove the excess nucleotide and reduce the salt content.

The pellet (80–90 mg) obtained in the above procedure was homogenized for 10–15 min in a tight Potter homogenizer in about 40 mL of 2 mM Tris-HCl, pH 7.6, containing 0.075–0.125 mM 8-azido-ATP. The solution was carefully protected from light and cooled in an ice bath. After homogenization, it was allowed to reach equilibrium for an additional 30 min and then an aliquot was removed in order to determine the amount of the exchanged nucleotide.

Covalent Labeling of Actin. G-Actin solution (35–45 mL at 2 mg/mL) containing 0.8–0.9 mol of 8-azido-ATP/mol of actin monomer was transferred to a beaker of about 60-mm diameter. The beaker was covered with a transparent poly(vinyl chloride) foil and placed in an ice bath, and the solution was flushed with a slow stream of N_2. After 15 min, the sample was irradiated for 8 min with a pair of 40-W low-pressure mercury lamps (Tungsram, Hungary) through the foil from a distance of 15 cm, under constant stirring. Immediately after irradiation, 50 μL of β-mercaptoethanol was added to quench the unreacted 8-azido-ATP.

In the case of F-actin, 8-azido-ATP–G-actin was polymerized by adding 50 mM KCl and 1 mM $MgCl_2$. After 1 h on ice, photolabeling was performed as with the G-actin.

After photolabeling, an aliquot was withdrawn for measurement of the extent of covalent incorporation, and the protein was precipitated by adding an equal volume of ice-cold 10% trichloroacetic acid. The precipitate was collected by centrifugation and washed once with cold 5% trichloroacetic acid and once with cold distilled water.

Peptide Analytical Methods. The precipitated protein was dissolved in about 20 mL of 8 M urea and 0.1 M Tris-HCl, pH 8.0, and reduction, carboxamidomethylation, and cyanogen bromide cleavage were performed according to Elzinga (1970).

Gel filtration of the cyanogen bromide peptides of actin and rechromatography of partially purified peptides were carried out on a 400 cm × 2 cm Sephadex G-50 column equilibrated with 25% acetic acid.

For separation of soluble and insoluble peptides, a 100 cm × 3 cm Sephadex G-10 column equilibrated with 0.01 M pyridine–acetic acid buffer, pH 6, was used, and the sample was applied in 70% formic acid, as described by Collins and Elzinga (1975).

For ion-exchange chromatography, a 0.8 × 12 cm SP-Sephadex column was used, and the column was developed with a gradient composed of 25% acetic acid and 25% acetic acid containing 3% pyridine.

Amino acid analyses were carried out on an instrument that employs single-column ion-exchange separation, with detection by post-column derivatization with ninhydrin. Its sensitivity limit is about 1 nmol. Sequences were determined by using both gas-phase and spinning-cup sequencers. The gas-phase sequencer (Applied Biosystems) was used with a load of about 1 nmol of peptide, and the PTH-amino acids were identified on a Hewlett-Packard Model 1090 HPLC, with a Zorbax cyanopropylsilane column (250 × 4.6 mm). In an effort to determine the site(s) of ATP binding, half of each sample from the Applied Biosystems sequencer was subjected to liquid scintillation counting. After a run was completed, the disk to which the sample was applied was also counted. The radioactivity always remained bound to the sample disk in the gas-phase sequencer. The labeled peptides were then run on a Beckman sequencer; about 20 nmol was applied, and the 0.1 M Quadrol program was used. The fractions were then divided in half for liquid scintillation counting and PTH-amino acid identification.

Miscellaneous Methods. Actin concentrations were determined by measurement of A_{280} and A_{290} (Houk & Ue, 1974) or, in the presence of azidonucleotide, by the dye-binding method of Bradford (1976).

To determine the exchanged nucleotide, an aliquot of actin solution was treated for 2 min with a $^1/_{10}$ volume of Dowex 1-X8 equilibrated with 2 mM Tris-HCl, pH 7.6. The resin was pelleted by brief centrifugation and the nucleotide content was measured in the supernatant by radioactivity.

Covalently bound nucleotide after photoactivation was measured from a sample precipitated and washed by 5% trichloroacetic acid on a Millipore filter.

Actin polymerization was measured by viscometry in an ice bath using an Ostwald-type viscometer having an outflow time of 38 s for water. The actin concentration was 1 mg/mL. Light-scattering measurements were carried out in a Brice-Phoenix OM 2000 light-scattering photometer with a 1-cm rectangular cuvette using a 546-nm filter. The actin concentration was 0.5 mg/mL. In each case, polymerization was induced by raising the salt concentration to 50 mM KCl and 1 mM $MgCl_2$.

Radioactivity measurements were made on a Beckman LS-100 liquid scintillation counter in Bray's cocktail.

Thin-layer chromatography was done on PEI–cellulose (Baker) with 0.6 M NaH_2PO_4 used as the developing solution.

Results

Preparation and Characterization of 8-Azido-ATP–G-Actin. The nucleotide binding constant of actin is about 100 times lower for ADP than for ATP (Neidl & Engel, 1979); therefore, the exchange of the bound nucleotide with 8-azido-ATP should be more effective from ADP–G-actin than from ATP–G-actin. In our hands the most effective way to prepare 8-azido-ATP–G-actin was to depolymerize pelleted ADP–F-actin in a low ionic strength buffer in the presence of 8-azido-ATP. This procedure gave consistently better results than the more circuitous preparation of ADP–G-actin followed by the exchange of ADP for 8-azido-ATP, probably because of the low stability of ADP–G-actin (Cooke & Murdoch, 1973).

To reduce the risk of unspecific labeling, the excess of 8-azido-ATP was kept as low as possible. In the presence of a

Table I: Exchange of the Bound Nucleotide of Actin for the 8-Azido Derivative[a]

actin	azidonucleotide bound (mol/mol of actin)
G-actin depolymerized from ADP–F-actin in a 2-fold excess of 8-azido-ATP	0.9
G-actin depolymerized from ADP–F-actin in a 2-fold excess of 8-azido-ATP and a 2-fold excess of ATP	0.2
F-actin polymerized from 8-azido-ATP–G-actin	0.9[b]
G-actin depolymerized from 8-azido-ADF–F-actin in 0.2 mM ATP	0.01

[a] Equal amounts of F-actin pellets were homogenized in a Teflon homogenizer in a low ionic strength buffer containing the nucleotide indicated for 10 min; after 30 min the bound nucleotide was determined as described under Experimental Procedures. The actin concentration was 2 mg/mL. [b] Measured from the resuspended pellet without depolymerization.

1.5–2 molar excess of 8-azido-ATP over actin, the extent of the exchange was usually 75–90%. If the depolymerization of F-actin was done in the presence of equal concentrations of 8-azido-ATP and ATP, with a twofold molar excess of each of the nucleotides, only 20% of actin contained 8-azido-ATP, indicating that ATP effectively competes with 8-azido-ATP in binding to actin. Data for a typical experiment are summarized in Table I.

Viscosity measurements showed that the rate and extent of polymerization of 8-azido-ATP–G-actin, protected from light was indistinguishable from that of ATP–G-actin. The critical concentration, calculated from the amount of the protein remaining in the supernatant of the polymerized actin samples, was 0.95 μM for ATP–G-actin and slightly higher, 2.4 μM, for 8-azido-ATP–G-actin. After polymerization there was no change in the azido–nucleotide content of actin, and the bound nucleotide analogue had been completely dephosphorylated to 8-azido-ADP, as shown by thin-layer chromatography on PEI–cellulose. The pellet obtained after the centrifugation depolymerized in the presence of 0.2 mM ATP with the simultaneous exchange of 8-azido-ADP to ATP (Table I).

Effect of Photolysis on ATP–Actin and 8-Azido-ATP–Actin. In order to determine the effect of UV irradiation of the polymerizability of ATP–G-actin, samples were irradiated, under conditions described under Experimental Procedures, for various lengths of time and then tested for polymerizability. It was found that for up to 10 min, there was no significant increase in the amount of the nonpolymerizable actin as determined by ultracentrifugation. Similar results were obtained by viscosity measurements; ATP–G-actin, irradiated for 8 min, showed essentially the same time course of increase in viscosity as control ATP–G-actin (Figure 1). (The amount of irradiation to which the 8-azido-ATP–actin was exposed was probably considerably greater than the minimum required for activation of the azido groups.)

Upon irradiation of 8-azido-ATP–G-actin (0.9 mol/mol of actin), a negligible change was found in the total azido-ATP content of actin (measured after Dowex 1 treatment). At the same time, 25–30% of the nucleotide became covalently bound to the protein (Table II).

The time course of the increase in the viscosity of irradiated 8-azido-ATP–G-actin shows that the final level of the viscosity is reduced about 20% and the rate of polymerization has also decreased (Figure 1). Similar results were obtained by monitoring the polymerization by light scattering (data not shown). After ultracentrifugation of the photolyzed and polymerized sample, a significant amount of protein remained in the supernatant. The distribution of the covalently bound nucleotide was the same in the pellet and the supernatant, indicating that there is no direct correlation between the loss of polymerizability and the covalent binding of the nucleotide analogue to the actin.

The extent of the incorporation did not depend on the state of actin since similar results were obtained if 8-azido-ATP–G-actin was first polymerized in the dark and then was irradiated in the F form.

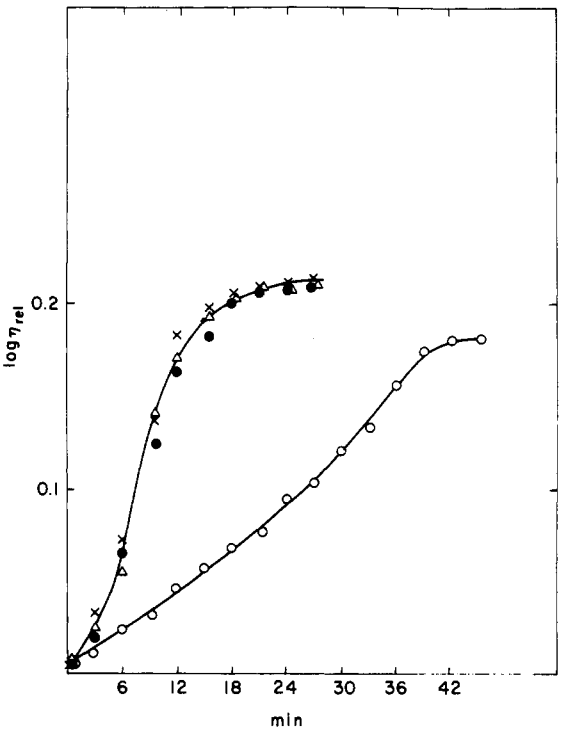

FIGURE 1: Effect of irradiation and photolabeling on polymerization of actin. Viscosity was measured as described under Experimental Procedures and assayed every 3 min. (●) ATP–G-actin; (△) ATP–G-actin illuminated for 8 min, (×) 8-azido-ATP–G-actin (in dark); (○) 8-azido-ATP–G-actin illuminated for 8 min.

Table II: Distribution of Polymerizable and Unpolymerizable Actin and Covalently Incorporated Nucleotide in 8-Azido-ATP–G-Actin Polymerized after Photoactivation[a]

actin sample	protein concn (mg/mL)	nucleotide incorporated (mol/mol of actin)
first cycle		
before centrifugation	1.85	0.28
supernatant	0.42	0.22
pellet (depolymerized)	1.5	0.25
second cycle		
before centrifugation	1.5	0.25
supernatant	0.5	0.23
pellet (depolymerized)	1.1	0.22

[a] G-Actin having bound 8-azido-ATP (0.9 mol/mol was photoactivated as described under Experimental Procedures. The sample was polymerized with 50 mM KCl and 1 mM $MgCl_2$. After centrifugation (150000g, 60 min) the pellet was depolymerized in 0.2 mM ATP, and the polymerization and the separation of polymerized and unpolymerized fraction were repeated in the second cycle.

Isolation of the Labeled Peptides and Identification of the Modified Amino Acids. In order to identify the amino acid residue(s) to which the photoactivated nucleotide analogue was

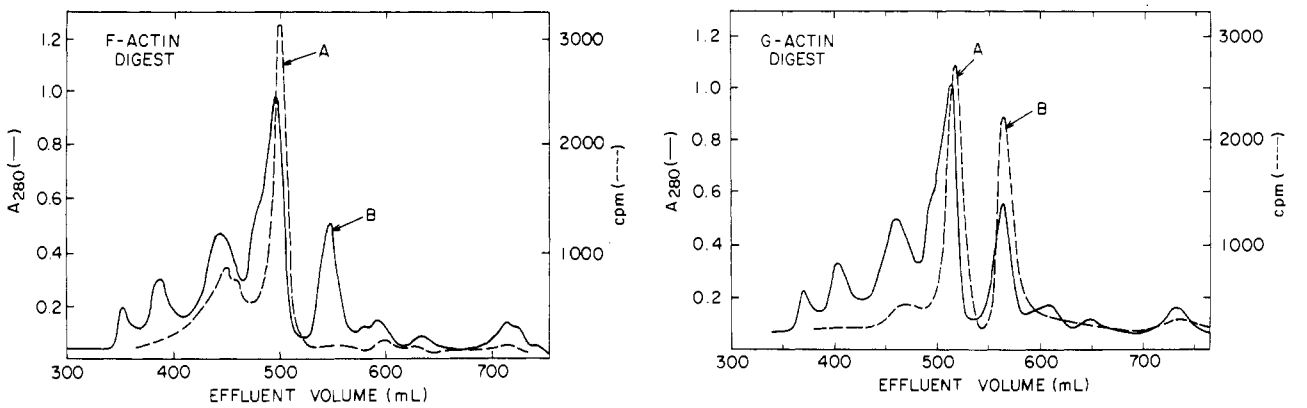

FIGURE 2: Elution profiles of CNBr peptides of photolabeled actin: left = F-actin; right = G-actin. A 400 × 2 Sephadex G-50 column was used and radioactivity was measured from 50-μL aliquots of the sample. Radioactive fractions were further purified as illustrated in Figure 3.

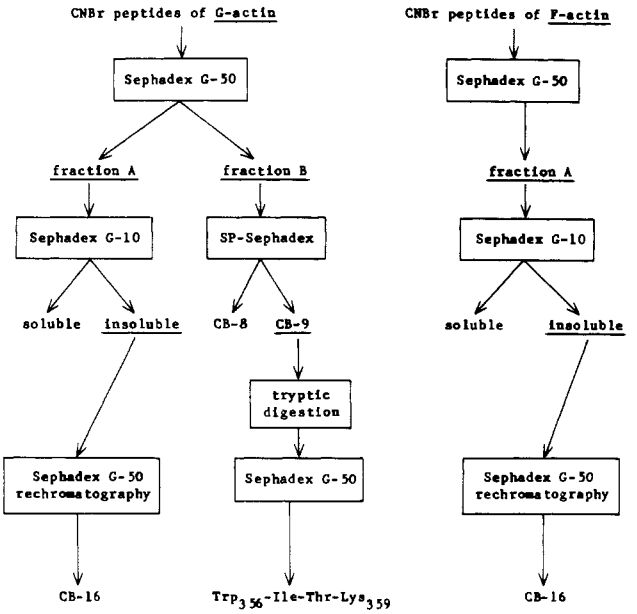

FIGURE 3: Summary of the steps employed in purifying labeled peptides from actin.

Table III: Amino Acid Composition of Two CB-16's Isolated from Labeled Actin[a]

	composition of CB-16	F-actin peptide	G-actin peptide
Lys	3	2.25	2.26
Arg	1	1.44	1.28
Asx	0	0.39	0.59
Thr	1	0.95	1.04
Ser	4	3.01	2.57
Glu	3	2.85	2.98
Pro	2	1.90	1.83
Gly	2	3.02	2.51
Ala	2	2.08	1.98
Val	1	1.24	1.48
Met	1	(1)	(1)
Ile	5	4.19	4.58
Leu	2	2.30	2.73
Tyr	1	0.98	1.52
Phe	1	1.07	1.22
Trp	1	0.70	0.65

[a] Since the peptides were isolated by gel filtration, low levels of contamination were expected, and their presence is reflected in the deviation from the expected integral ratios, particularly for Asx, Gly, and Leu. Tryptophans were determined after hydrolysis in methanesulfonic acid (Simpson et al., 1976), and homoserine (from methionine) was not quantified.

bound, 2 μmol of actin containing 0.8–0.9 mol of 8-azidonucleotide per actin monomer was irradiated by UV light (see

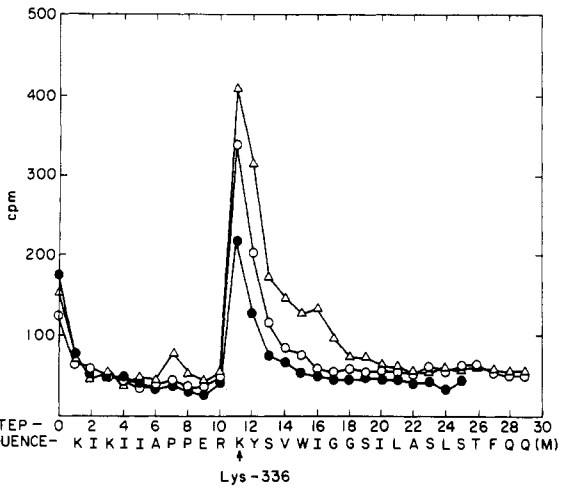

FIGURE 4: Beckman 890c sequencer runs of peptide CB-16. At each step, one-eighth of each sample was counted, and the remainder was analyzed for the PTH-amino acid present. The 0 step was carried out without addition of PITC, and the counts observed represented washout of unbound [^{14}C]ADP. The appearance of counts in steps 12–17 was probably caused by the fact that the phosphate(s) remaining on the PTH–Lys to which the labeled convalently bound nucleotide was bound renders the PTH sparingly soluble in the organic solvents used to extract the PTH intermediates from the spinning cup. The sequence of CB-16 (residues 326–355 in actin) is shown at the bottom in single-letter code. The curve labeled Δ was from an F-actin peptide; 175 nmol containing 51 000 cpm was applied. The curve labeled O was also from F-actin; 100 nmol containing 26 000 cpm was applied. The curve labeled ● was from a G-actin peptide; 100 nmol containing 24 000 cpm was applied.

Experimental Procedures). The labeling was carried out with both G- and F-actin. Following the removal of nonbound nucleotide, the protein was reduced, carboxamidomethylated, and subjected to cyanogen bromide treatment. Cyanogen bromide peptides were separated on a Sephadex G-50 column in 25% acetic acid. Typical elution profiles are shown in Figure 2. Inspection of the elution profile indicates that UV irradiation has not altered the methionines and tryptophans significantly since the elution curve observed was similar to that which had been seen earlier.

Whenever actin was irradiated in the F-form, only a single major radioactive fraction was obtained on the G-50 column (fraction A). Separation of this fraction on Sephadex G-10 in neutral buffer followed by rechromatography on G-50, again in 25% acetic acid, indicated that the peptide which contained the radioactive label was CB-16. Table III shows the amino acid analyses of the nucleotide-containing peptides obtained in these different experiments. By automatic Edman degradation, the radioactivity was detected in step 11, which cor-

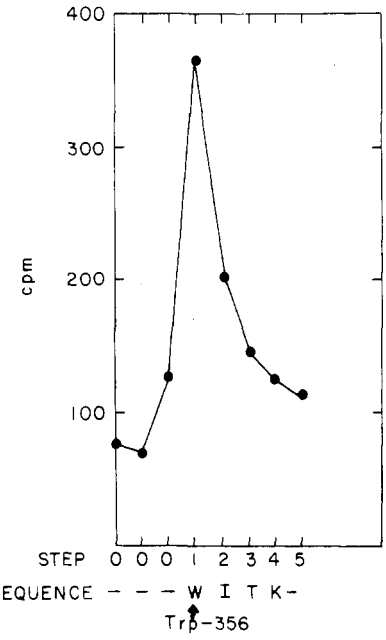

FIGURE 5: Sequence analysis of the labeled tetrapeptide Trp-Ile-Thr-Lys isolated from CB-9 of G-actin. The composition was Trp, 0.2; Ile, 1.0; Thr, 0.8; Lys, 1.1. Fifty nanomoles containing 7000 cpm was applied; one-third of each step was counted. Three "0 cycles", without PITC were run. As with CB-16, radioactivity appeared in steps subsequent to the location of the labeled residue.

responds to Lys-336 (Figure 4).

A somewhat more complicated picture was obtained when 8-azido-ATP–G-actin was irradiated. The major radioactive peptide in all cases was also CB-16 and the labeled residue was Lys-336; however, in four experiments out of ten, a second radioactive peak was also observed (fraction B). This region is known to contain CB-8 and CB-9. It was pooled and chromatographed on SP-Sephadex C25; the radioactive peak was then digested with trypsin, and the digest was chromatographed on Sephadex G-50. Radioactivity was coeluted with a tetrapeptide whose sequence is Trp-Ile-Thr-Lys. The labeled residue in this peptide was Trp-356 (Figure 5).

Discussion

As can be seen from the data of Table I, under the experimental conditions described here it is possible to prepare G-actin, which contains an almost stoichiometric amount of 8-azido-ATP. The analogue seems to bind at the nucleotide binding site, since ATP effectively competes with the binding of 8-azido-ATP. G-Actin containing 8-azido-ATP as the bound nucleotide retains its polymerizability, and the bound azidonucleotide triphosphate is completely dephosphorylated to the diphosphate during the polymerization. These findings indicate that actin belongs to that group of proteins which can accept 8-azido-ATP, an analogue of ATP, containing a bulky substituent at position 8 in the purine ring.

Upon mild UV irradiation, under conditions that are not deleterious for actin in the absence of the photolabel, about 25% of the analogue becomes covalently bound to actin. The extent of the covalent incorporation is independent of the state of actin during the irradiation, i.e., irradiation of 8-azido-ATP–G-actin and of 8-azido-ADP–F-actin gave similar degrees of incorporation.

The relatively high yield of the incorporation made it possible to isolate the peptides to which the radioactive nucleotide analogue is bound. Using cyanogen bromide fragmentation and the peptide separation procedures described earlier (Elzinga, 1970), we have found the label in CB-16, the penultimate C-terminal cyanogen bromide peptide, in both G- and F-actin. Sequence analysis of CB-16 revealed that the label is bound to Lys-336 (Figure 4).

In several cases, an additional site was also labeled when the reaction was carried out with G-actin. The amount of radioactivity incorporated into this peptide was 25–40% of the total, and further analysis showed that the residue to which the label was bound is Trp-356 (Figure 5). The reason for the variation in the pattern of labeling of G-actin is not clear. Perhaps G-actin can exist in two slightly different conformations and the proximity of Lys-336 and Trp-356 to the azido group of 8-azido-ATP is different in the two conformations. In one conformation, Lys-336 could be adjacent to the azido group, while in the other, both Lys-336 and Trp-356 could be equidistant from the group. We do not know what conditions could cause this apparent difference in conformation since the variation G-actins that labeled differently were prepared by using identical procedures. Whatever the explanation for the difference in labeling, it seems likely that Lys-336 and Trp-356 are close to each other and to the nucleotide ring in the tertiary structure of actin.

Other studies of the nucleotide binding site of actin include one by Faust et al. (1974) in which actin was covalently linked to 6-[(dinitrophenyl)thio]-ATP; while some incorporation was achieved, the residues to which the label was bound were not identified. Recently, Mahoney (1985) reported incorporation of 8-azido-ADP into F-actin with an efficiency of 4–5% and a tentative incorporation into "a C-terminal peptide", and Kuwayami and Yount (1986) have reported that 2-azido-ATP incorporated into actin binds covalently to Tyr-306.

Lu and Szilagyi (1981) have studied the reactivities of the lysines in actin under various conditions. They reported that the reactivity of Lys-336 increased substantially upon polymerization of G-actin–ATP to F-actin–ADP and suggested that this increased reactivity was due to the loss of the terminal phosphate of ATP which was proposed to lie in the vicinity of the ϵ-amino group of Lys-336. The geometrical relationship between the nucleotide and Lys-336 is not yet clear, but our results are consistent with the conclusions of Lu and Szilagyi.

Preliminary X-ray diffraction studies on an actin–DNase complex (Suck et al., 1981; Sakabe et al., 1983; Kabsch et al., 1985) show that the actin molecule consists of a larger and a smaller domain forming a two-lobe structure, with the ATP-binding site tentatively placed in the cleft between the two lobes. Mornet and Ue (1984) suggested that the smaller lobe contains the N-terminal part of actin. The N-terminal 68 residues may play an essential role in the binding of the divalent cation (Jacobson & Rosenbusch, 1976), and it must be noted that physicochemical studies (Loscalzo & Reed, 1976; Brauer & Sykes, 1982; Miki & Wahl, 1985) indicate that the binding sites of divalent cation and of the nucleotide are close together on the surface of actin.

Vanderkerckhove et al. (1985) have carried out studies analogous to those described here using affinity-labeling derivatives of phalloidin and have found that an alkylating (iodoacetyl)phalloidin binds to Met-119 and Met-355. The latter is adjacent to Trp-356, one of the residues labeled by 8-azido-ATP. Since phalloidin and the nucleotide bind simultaneously to actin, but label adjacent side chains, they must occupy sites that are close together on the surface of actin. Suck et al. (1981) have suggested that the nucleotide binds in the cleft between two lobes of actin, and Vandekerckhove et al. (1985) suggest that phalloidin also binds in this cleft.

Fluorescence energy transfer measurements have been used to determine the distance between Cys-374 and the nucleotide

and the divalent metal binding site(s) of actin. The distance between Cys-374 and the nucleotide was found to be about 3 nm (Miki & Mihashi, 1978; Miki & Wahl, 1984; Dos Remedios & Cooke, 1984), and a very similar value (3.2 nm) was obtained for the distance between the divalent cation and Cys-374 (Miki & Wahl, 1985). The data presented here further our knowledge of the nucleotide binding site by demonstrating the involvement of Lys-336 and Trp-356. As the crystal structure of actin is refined to higher resolution, our overall understanding of the structure of actin will become more clear, and the results reported here can be used to fill in the picture. For example, the binding of Trp-356 to 8-azido-ATP in G-actin but not in F-actin may signal a change in the conformation of actin in this region.

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Registry No. L-Lys, 56-87-1; L-Trp, 73-22-3; 8-azido-ATP, 53696-59-6; [^{14}C]-8-azido-ATP, 103934-14-1.

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