The Reactivity and Function of Thiol Groups in Trout Actin

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1. Considerable differences were found between the rates and degrees of modification of native trout actin with iodo[2-¹⁴C]acetate and iodo[1-¹⁴C]acetamide. 2. With iodoacetate, G- and F-actin were both labelled in the *N*-terminal peptide only. This modification had little effect on the ability of the actin to polymerize. 3. Iodoacetamide labelled three cysteine residues in both G- and F-actin. The modified cysteine residues were identified from the position of the corresponding tryptic peptides on peptide 'maps'. 4. The modification had little effect on the ability of G-actin to polymerize, to bind ATP or to bind Ca²⁺, or on the ability of F-actin to depolymerize. 5. It is concluded that the three cysteine residues present on the 'surface' of the native trout actin molecule have no direct role in the polymerization processes, the binding of ATP, or the binding of Ca²⁺.

Work on the role of cysteine residues in actin function was hampered initially by the lack of an accurate molecular weight value, and hence an accurate value for the number of cysteine residues present. Rees & Young (1967) prepared pure aggregate-free rabbit actin and obtained a value of 46000 for the molecular weight. This value has now been confirmed by peptide analysis (Adelstein & Kuehl, 1970; Elzinga, 1970), and corresponds to five cysteine residues/molecule (Adelstein & Kuehl, 1970; Elzinga, 1970; Johnson & Perry, 1968). Radioactivity-incorporation studies revealed a similar number of cysteine residues in trout actin (Bridgen, 1971).

The role of thiol groups in the protein is uncertain. It has been postulated that they are involved in the polymerization of G-actin (Kuschinsky & Turba, 1962; Barany, 1956; Katz & Mommaerts, 1962; Drabikowski & Gergely, 1963), nucleotide binding (Strohman & Samorodin, 1962; Katz, 1963; Kuehl & Gergely, 1969), and myosin combination (Perry & Cotterill, 1964; Bailin & Barany, 1967). Seraydarian *et al.* (1968), however, found no effect on the ability of rabbit G-actin to polymerize or bind myosin after modification of two or three of the cysteine residues with 2-aminoethylisothiuronium. Similar results were obtained by Lusty & Fasold (1969) after modification of three accessible thiol groups in rabbit actin with an azo-dye.

The present paper examines the effect of specific modification of thiol groups on the ability of trout G-actin to polymerize, to bind ATP and to bind Ca²⁺. The modified thiol groups are identified on peptide 'maps' and correlated with the cysteine-peptide sequences reported by Bridgen (1971).

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Materials and Methods

Materials

2-Mercaptoethanol, iodoacetic acid, iodoacetamide and trypsin (twice-recrystallized) were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Solutions of iodoacetic acid were dried *in vacuo* before use to remove free iodine. Iodoacetamide was recrystallized from hot benzene. Iodo[2-¹⁴C]acetic acid was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and diluted with carrier iodoacetic acid to a specific radioactivity of 0.2 mCi/mmol before use. Iodo[1-¹⁴C]acetamide was purchased from the same source and diluted with carrier iodoacetamide to a specific radioactivity of 1.0 mCi/mmol before use.

Methods

Preparation and enzymic digestion of the protein. The preparation and determination of trout actin, tryptic digestion, and peptide 'mapping' of carboxymethylated actin were performed as described by Bridgen (1971). A molecular weight of 45000 (Adelstein & Kuehl, 1970) for the native protein was assumed throughout.

Chemical modification of native actin. Alkylation of thiol groups in the native protein was done at room temperature (20°C) in 0.1 M-NH₄HCO₃ buffer, pH 8.0. Reaction mixtures contained 5 mg of protein/ml and 5 mM-iodoacetamide or 5 mM-sodium iodoacetate. Reactions were stopped after the required time by adding 2-mercaptoethanol to a final concentration of 25 mM, and the alkylated protein was then isolated by gel filtration on a column (52 cm × 1.2 cm) of Sephadex G-25 eluted with 0.5 M-acetic acid. Incorporation of radioactivity into the protein was measured as described

by Bridgen (1971). Protein controls, containing all the reagents except the alkylating agents, were prepared and maintained under conditions of pH and temperature identical with those of the test solutions.

Measurement of viscosity. Ostwald-type viscometers of 1.5 ml capacity and water-outflow time of 65 s at 25°C were used. Solutions were allowed to equilibrate at this temperature during the poly-

Fig. 1. Rate of incorporation of radioactivity into native trout G- and F-actin

Native G-actin (a) or F-actin (b) ($1 \mu\text{mol}$) was incubated with iodo[2- ^{14}C]acetate ($50 \mu\text{Ci}$, pH 8.0) for 30 min. Samples ($5 \mu\text{l}$) were counted for radioactivity.

Fig. 2. Rate of incorporation of radioactivity into native trout G- and F-actin

Native G-actin (a) or F-actin (b) ($1 \mu\text{mol}$) was incubated with iodo[1- ^{14}C]acetamide ($25 \mu\text{Ci}$, pH 8.0) for 30 min. Samples ($5 \mu\text{l}$) were withdrawn and counted for radioactivity.

merization process. Polymerization was induced by adding 0.1 vol. of 1.0M-KCl-0.5M-tris-HCl buffer, pH 7.5, to the actin solution.

Assay of bound ATP. Protein samples (100 μl) were removed at 5 min intervals during the alkylation procedure. Free nucleotides and cations were adsorbed on Dowex-1 and Dowex-50 respectively (Strzelecka-Golaszewska & Drabikowski, 1967) and the protein was then precipitated by addition of 0.1 vol. of ice-cold 6M-HClO₄. The concentration of bound nucleotide was evaluated from the E₂₆₀ of the perchloric acid supernatant by using ε 147001·mol⁻¹·cm⁻¹ (Bock *et al.* 1956).

Determination of Ca²⁺. Ca²⁺ was determined by a modification of the displacement method of Hilde-

brand & Reilley (1957). Samples from the nucleotide assays were neutralized with 4M-NaOH, and 1M-NH₄Cl-NH₃ buffer, pH 9.5 (100 μl), was added. To each sample were then added 500 μl of a Mg²⁺-EDTA complex [EDTA (0.301 g) and MgSO₄·7H₂O (0.199 g) diluted to 100 ml] and two drops of an Eriochrome Black T indicator solution (0.2 g of indicator dissolved in triethanolamine-ethanol, 3:1, v/v). After mixing, titration against 0.1 M-EDTA was performed, the end-points being determined from the E₅₇₀ of the mixture, this being the extinction maximum of the final complex.

Table 1. *Amino acid analyses of the three radioactive peptides eluted from the peptide 'map' of carboxymethylated native actin*

Results are expressed as molar ratios relative to alanine for peptide 1', aspartic acid for peptide 2', and phenylalanine for peptide 3'. Abbreviation: CMCys, S-carboxymethylcysteine.

Amino acid	No. of residues		
	Peptide 1'	Peptide 2'	Peptide 3'
CMCys	0.4	0.5	0.7
Asp	1.8	2.0	—
Thr	1.7	—	—
Ser	1.8	—	—
Glu	2.1	—	—
Gly	2.3	—	—
Ala	2.0	—	—
Val	1.7	—	—
Ile	—	1.7	—
Leu	1.9	—	—
Phe	—	—	1.0
Lys	0.9	—	—
Arg	—	1.2	—

Table 2. *Amino acid analyses of chymotryptic peptide C-5 (Bridgen, 1971) and chymotryptic peptide 3 (Lusty & Fasold, 1969)*

Results are expressed as molar ratios relative to leucine. Abbreviation: CMCys, S-carboxymethylcysteine.

Amino acid	No. of residues	
	Peptide C-5	Peptide 3
CMCys	0.7	0.8
Thr	0.8	0.9
Ser	0.7	1.6
Glu	—	0.9
Pro	0.9	1.0
Leu	1.0	1.0
Phe	1.1	—
Arg	1.2	0.8

Fig. 3. *Peptide 'map' of a tryptic digest of native trout G-actin modified with iodo[1-¹⁴C]acetamide*

The open spots represent peptides present in the tryptic digest; shaded spots were radioactive. Spots 1, 2 and 3 were present on a 'map' of carboxymethylated denatured trout actin (Bridgen, 1971) but were absent from this map. Spots 1', 2' and 3' were the only new peptides to appear; spot 1' was ninhydrin-negative. After application of peptide material (1 mg) at 'O', descending chromatography was performed with butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) along the axis labelled BAWP. Electrophoresis was then performed at right angles in pH 6.5 buffer [10 % (v/v) pyridine, 0.3 % (v/v) acetic acid] at 60 V/cm for 1 h, with an aspartic acid marker, A.

Results and Discussion

Native G- and F-actin from trout both incorporated radioactive iodoacetate equivalent to the modification of one cysteine residue/mol of protein

(Figs. 1a and 1b). Reaction with F-actin was 1.8 times slower than that with G-actin. Tryptic peptide 'maps' prepared from the modified actins showed that radioactivity was confined to the same spot in both cases. This spot was ninhydrin-negative and from its

Fig. 4. Assay of released Ca^{2+} , released ATP and loss of ability of native trout G-actin to polymerize on modification of thiol groups with iodoacetamide

□, Ca^{2+} released; △, ATP released; ●, specific viscosity; ○, number of modified residues calculated from the incorporation of radioactivity (see Fig. 2a). Results for the Ca^{2+} and ATP assays are expressed by assuming 1 mol of each to be present/mol of untreated protein.

Fig. 5. Assay of released Ca^{2+} , released ATP and loss of ability of an untreated G-actin protein control to polymerize
□, Ca^{2+} released; △, ATP released; ●, specific viscosity. The Ca^{2+} and ATP assays assumed 1 mol of each to be present/mol of protein at zero time.

position on the 'map' could be identified as the putative *N*-terminal peptide (Bridgen, 1971). Alkylation with iodoacetamide proceeded considerably faster than the reaction with the free acid, with no appreciable difference in reactivity between the G- and F-actins (Figs. 2*a* and 2*b*). Both forms incorporated an amount of label equivalent to the modification of three thiol groups. The tryptic peptide 'maps' of the carboxamidomethylated G- and F-actins both showed three strongly radioactive spots, one of which (peptide 1') was ninhydrin-negative (Fig. 3). The radioactive peptides were eluted and amino acid analysis gave the compositions shown in Table 1. Comparison with the results of Bridgen (1971) indicated that the three modified thiol groups were those from the *N*-terminal and C-terminal regions of the protein, and also that present in the tryptic peptide Cys-Asp-Ile-Asp-Ile-Arg. Hence, these three thiol groups appear to be located at or near the surface of the protein. Lusty & Fasold (1969) isolated two peptides analogous to peptides 2' and 3' (Table 1) from chymotryptic digests of native rabbit actin labelled with 2,2'-dicarboxy-4'-iodoacetamidobenzene. However, these authors found that peptide 1' was labelled only in the denatured protein, and their third labelled peptide isolated from the native protein appears similar (Table 2) to the chymotryptic peptide C-5 obtained by Bridgen (1971). This difference in reactivity may be due to a difference either in the protein structure or in the alkylating agent.

The effect of modification by iodoacetamide on the binding of Ca^{2+} and ATP by G-actin, and its ability to polymerize, are shown in Fig. 4. Approx. 30% of the bound nucleotide was lost after incubation with the reagent for 30 min. The rate of loss was linear and was not the same as the labelling rate of the protein. Hence alkylation of these three cysteine residues appeared to have no direct effect on the binding of ATP to the protein. As modification of cysteine residues could produce a conformational change, which in turn could destabilize the ATP-binding site, thus causing a slow release of bound ATP, the ATP bound to an unmodified G-actin protein control was assayed (Fig. 5). The rate and degree of loss of bound nucleotide were only slightly less than in the modified protein. Thus the observed release of ATP appears to be an independent effect caused by maintaining the protein in an ATP-free solvent (Asakura, 1961). As release of bound ATP produces a concomitant release of Ca^{2+} (Maruyama & Gergely, 1961; Barany *et al.*, 1962), a similar explanation almost certainly applies to the release of the Ca^{2+} . The reversal of the release rates of the ATP and Ca^{2+} between the modified protein and the protein control was probably due to greater error in the Ca^{2+} determinations.

The rate of loss of the ability of both the actin

control and the modified actin to polymerize was very similar to the rate of loss of ATP and Ca^{2+} . Under normal conditions, G-actin that has lost its bound ATP or Ca^{2+} will not polymerize (Martonosi & Gouvea, 1961) and it appears that the loss of polymerizing ability was a secondary effect resulting from the loss of bound ATP. The F-actin, with the same three cysteine residues modified, could be depolymerized to G-actin as judged by the qualitative loss of birefringence of the F-actin solution on addition of ATP (0.2 mM).

From the evidence presented here the role of cysteine residues in the ability of actin to polymerize, and to bind nucleotide and calcium, would appear to be a small and indirect one. Possibly thiol groups stabilize binding sites on actin or play a part in maintaining the overall protein structure.

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