Crystallization of cytoplasmic actin in complex with deoxyribonuclease I

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Crystals of cytoplasmic (porcine liver) actin in complex with deoxyribonuclease I (DNAase I) were prepared for structural determination by X-ray-diffraction analysis. The crystallization of porcine liver actin–DNAase I complex is preceded by a brief treatment with immobilized trypsin, whereby a C-terminal tri- or di-peptide including cysteine-374 is removed from the actin without any noticeable degradation of both proteins as judged by sodium dodecyl-sulphate/polyacrylamide-gel electrophoresis. Analysis of the crystals obtained does not reveal any differences in the three-dimensional structure of porcine liver actin from its skeletal compartment at up to 0.6 nm resolution. However, in contrast with crystalline skeletal-muscle actin–DNAase I complex, heavy-atom substitution of crystals of porcine liver actin–DNAase I complex could not be achieved with methyl mercuriacetate. Evidence is presented that, in porcine liver actin, the N-terminal cysteine residue is not located at position no. 10, as in skeletal- and smooth-muscle actin, but most probably at position no. 17. Thus, because this site is covered by DNAase I, the cysteine becomes inaccessible to titration with 5,5'-dithiobis-(2-nitrobenzoic acid) after complex-formation with DNAase I.

Actin is one of the most ubiquitous proteins in nature. It is present in all eukaryotic cells, and it participates in a number of different cellular activities such as locomotion, endo- and exocytosis, and cytokinesis (for a review see Korn, 1982). Isoenzymic variants of actin have been discerned, differing in their isoelectric points (Wahlen *et al.*, 1976; Rubenstein & Spudich, 1977). Actin isolated from sarcomeric muscle cells represents the most acidic isoform (α -actin), in smooth-muscle and in non-muscle cells two different isoforms (β - and γ -actin) have been shown to co-exist (Wahlen *et al.*, 1976; Rubenstein & Spudich, 1977; Garrels & Gibson, 1976). Therefore within mammalian eukaryotic cells the variability of actin isoforms is tissue- rather than species-specific.

Among the eukaryotic actins the most divergent

isoforms are α -actin (sarcomeric actin) and the (β - and γ -) cytoplasmic actins, which differ by at least 25 amino acid residues (Vandekerckhove & Weber, 1978*a,b*). It has been reported that cytoplasmic and sarcomeric actins differ in their critical concentrations of polymerization (Gordon *et al.*, 1976*a*) and their abilities to activate the Mg^{2+} -dependent myosin ATPase (Gordon *et al.*, 1976*b*). Since the three-dimensional structure of rabbit skeletal-muscle actin in complex with bovine pancreatic deoxyribonuclease I (DNAase I) is known at low resolution (Suck *et al.*, 1981) and is in the process of being analysed at high resolution by X-ray-diffraction techniques, it was deemed necessary to supplement this information by an analysis of the three-dimensional structure of cytoplasmic actin. Such a comparison of the two actins should yield valuable information about the structural similarities of presumed functional domains of both isoforms. Here we report the crystallization of cytoplasmic actin (isolated from porcine liver) in complex with bovine pancreatic DNAase I and present preliminary X-ray-diffraction data of the crystals obtained.

Abbreviations used: i.e.f., isoelectric focusing; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); MeHgAc, methyl mercuriacetate; NEM, N-ethylmaleimide; DNAase I, deoxyribonuclease I; SDS, sodium dodecyl sulphate.

Experimental

Protein preparations

Bovine pancreatic DNAase I (EC 3.1.21.1) was obtained commercially from Worthington and further purified by chromatography on hydroxyapatite as described previously (Mannherz *et al.*, 1980). Actin from rabbit skeletal muscle was prepared from acetone-dried powder, by a method that included two cycles of polymerization and depolymerization (Mannherz *et al.*, 1980). Actin from porcine liver was purified by a slight modification (Jaberg, 1983), and from porcine brain as described by Gordon *et al.* (1967b). All protein preparations were found to be at least 95% pure as judged by polyacrylamide-gel electrophoresis in the presence of SDS. The complex of DNAase I and actin was obtained by mixing both purified proteins at an equimolar ratio. Non-complexed material was removed by gel filtration over an Ultrogel AcA 44 (LKB, Frankfurt, Germany) column (dimensions: 50 cm high, 4 cm diameter) equilibrated with buffer A (0.5 mM-Na₃/0.1 mM-CaCl₂/5 mM-Hepes, pH 7.4).

Enzymic activity

DNAase I activity was determined by the hyperchromicity assay developed by Kunitz (1950) and with the use of a Shimadzu UV-300 spectrophotometer as described by Mannherz *et al.* (1980).

Electrophoretic techniques

Polyacrylamide-gel electrophoresis in the presence of SDS was performed as described by Laemmli (1970). I.e.f. was performed in cylindrical gels and extended to two-dimensional analysis as described by O'Farrell (1975). For the analysis of radioactivity of labelled protein after SDS/polyacrylamide-gel electrophoresis, the gels were cut into slices of 5 mm width, homogenized in 2 ml of 2% (w/v) SDS and mixed with 10 ml of scintillation cocktail 808E (Riedel de Haen, Seelze-Hannover, Germany).

Miscellaneous

Labelling of actin with [¹⁴C]NEM was performed as described by Lusty & Fasold (1969). Unchanged label was removed from protein by gel filtration over a Sephadex G-50 column (dimensions: 50 cm high, 1.5 cm diameter). Protein concentrations were determined as described by Bradford (1976), with bovine serum albumin as standard. Trypsin was immobilized on CNBr-activated Sepharose 4B following the protocol given by the manufacturer.

Crystallization

Crystals of porcine liver actin-DNAase I complex were obtained in 0.1 M-imidazole

(pH 6.7)/0.1 mM-CaCl₂/1 mM-MgATP/0.1 mM-BaCl₂, with 10% (w/v) poly(ethylene glycol) 6000 as precipitant. Before crystallization actin-DNAase I complex was treated with immobilized trypsin for 2 h at room temperature.

Materials

Trypsin [EC 3.4.21.4; tosylphenylalanylchloromethane ('TPCK')-treated] was obtained commercially from Worthington and used without further purification. CNBr-activated Sepharose was obtained from Pharmacia, Freiberg, Germany. [¹⁴C]NEM was a commercial product of Amersham Buchler, Braunschweig, Germany. All other reagents were of analytical grade.

Results

Crystallization of cytoplasmic actin in complex with DNAase I

Fig. 1 gives an analysis of rabbit skeletal-muscle and porcine brain and liver actin on SDS/polyacrylamide-gel electrophoresis and i.e.f. On SDS/polyacrylamide-gel electrophoresis, all three actins exhibit an identical apparent molecular mass, whereas differences between skeletal and cytoplasmic actins appear after i.e.f. Both cytoplasmic actins exhibit an identical isoenzymic pattern after i.e.f., i.e. both are composed of apparently identical ratios of β - and γ -actin (Fig. 1).

Crystals suitable for X-ray-diffraction analysis were obtained after 8 weeks (Fig. 2). The crystals contain an enzymically active actin-DNAase I complex that was verified after extensive washing of several crystals (Table 1). The progress curve of DNA degradation by dissolved crystals exhibits the sigmoidal time-dependence typical for the actin-inhibited DNAase I activity. Washed and dissolved crystals were analysed by SDS/polyacrylamide-gel electrophoresis and two-dimensional gel

Table 1. Analysis of the DNA-degrading activity of solutions employed in successive washing steps of crystals of porcine liver actin-DNAase I complex and of crystals dissolved in buffer A

Activity is expressed in total Kunitz (1950) units (1 KU equals the change in absorbance at 260 nm of 0.001/min).

Supernatant of washing step no.	Total DNAase I activity in KU
1	2438
2	338
3	47
4	16
Dissolved crystals	2100

Fig. 1. Analysis of rabbit skeletal-muscle, porcine brain and liver actin by SDS/polyacrylamide-gel electrophoresis and i.e.f. Slot (a), skeletal, (b) porcine brain and (c) porcine liver actin after SDS/polyacrylamide-gel electrophoresis. I.e.f. on cylindrical gels of : (d) skeletal; (e) porcine brain; and (f) liver actin; (g) co-migration of (e) and (f); and (h) of (d) and (f).

electrophoresis. SDS/polyacrylamide-gel electrophoresis revealed the simultaneous presence of two bands corresponding to actin and DNAase I. Analysis of the last washing solution before dissolving the washed crystals did not reveal any protein bands after SDS/polyacrylamide-gel electrophoresis. Two-dimensional gels (first dimension i.e.f.) showed the occurrence of various DNAase I isoforms and β - and γ -actin within the crystals (results not shown).

No crystal growth could be induced when DNAase I was complexed with porcine brain actin. The reason for this difference is not known at present.

Analysis of the thiol reactivity of cytoplasmic actin in complex with DNAase I

Cytoplasmic actins differ from their sarcomeric counterpart by at least 25 amino acid residues (Vandekerckhove & Weber, 1978a,b). Rabbit skeletal-muscle actin contains five cysteine residues; cytoplasmic actins contain six (in the *N*-terminal region a shift of cysteine-10 to position 17 and an additional cysteine residue at position 271; Vandekerckhove & Weber, 1978b). Previous stu-

dies have implicated that, after trypsin treatment of skeletal-muscle actin-DNAase I complex before crystallization, a C-terminal oligopeptide including cysteine-374 is removed from actin, whereas its *N*-terminus remains intact, owing to its interaction with DNAase I (Konrad, 1977; Mannherz *et al.*, 1980). Since this treatment was found essential for obtaining crystals of cytoplasmic actin in complex with DNAase I, an analysis of the effect of trypsin treatment on the reactivity of the thiol groups of actin was undertaken. DNAase I does not contain any free thiol groups (Liao *et al.*, 1973). Complexes of rabbit skeletal-muscle and of porcine liver actin with DNAase I at $5\mu\text{M}$ were treated separately with a 200-fold molar excess of NEM supplemented with [^{14}C]NEM for 4h at pH 7.4 and at room temperature. This reagent preferentially labels the C-terminal cysteine residue (position 374) of rabbit skeletal-muscle actin (Lusty & Fasold, 1969). The thus-treated actin-DNAase I complexes were freed from unchanged NEM by passage over a Sephadex G-50 column (dimensions: 30cm high and 1.5cm diameter) equilibrated with buffer A. From the analysis of the radioactivity in the protein fractions the molar ratio of covalently

Fig. 2. Crystals obtained from porcine liver actin-DNAase I complex
The horizontal bar represents 0.1 mm.

attached label was calculated to be 1.2 and 1.6 for skeletal actin-DNAase I and liver actin-DNAase I complex respectively. After SDS/polyacrylamide-gel electrophoresis the radioactivity was found to be confined to the actins of both complexes. Both labelled complexes were then treated with immobilized trypsin as described above and again subjected to gel filtration over the Sephadex G-50 column. No radioactivity co-migrating with the protein peaks could be determined for skeletal actin-DNAase I complex, whereas the radioactivity in the protein fraction of liver actin-DNAase I complex was reduced to about 30% (Fig. 3). After SDS/polyacrylamide-gel electrophoresis of this material no obvious degradation of its protein constituents was observed, and in a subsequent analysis of gel slices the residual radioactivity was found to be still associated with liver actin (results not shown). Independently, the reactivity of cysteine residues in porcine liver and rabbit skeletal-muscle actin before and after complexing with DNAase I was probed by treating both actins

(at 5 μM) with 10 μM -Nbs₂ as described previously (Mannherz *et al.*, 1983). The numbers of thiol groups available for Nbs₂ were found to be 1.6 and 2.4 before, and 1.2 and 1.6 after, complexing with DNAase I for rabbit skeletal and porcine liver actin respectively.

Crystallographic data of crystalline cytoplasmic actin-DNAase I complex

The crystals obtained from porcine liver actin-DNAase I complex have the same appearance as the type III crystals of skeletal-muscle actin-DNAase I complex (Mannherz *et al.*, 1977; Suck *et al.*, 1981). Crystallographic analysis reveals that they are also of identical space group ($P2_12_12_1$) and of identical unit-cell dimension [$a = 13.3 \text{ nm}$ (133 Å), $b = 5.64 \text{ nm}$ (56.4 Å), and $c = 11 \text{ nm}$ (110.0 Å)] (Suck *et al.*, 1981). The diffraction limit of native crystals obtained from porcine liver actin-DNAase I complex was found to be the same as from crystalline skeletal-muscle actin-DNAase I complex [about 0.25 nm (2.5 Å); Suck *et al.*, 1981].

Fig. 3. Analysis of [^{14}C]NEM-labelled actin-DNAase I complex by gel filtration over Sephadex G-50 before and after trypsin treatment

(a) Elution of [^{14}C]NEM-labelled rabbit skeletal muscle actin-DNAase I complex before, and (b) after, trypsin treatment; (c) and (d) identical analysis of porcine liver actin-DNAase I complex. The fraction size was 3 ml; ●, A_{280} ; ○, radioactivity (c.p.m.)/0.1 ml aliquot. The second peak in (a) and (c) represents unchanged NEM.

however, no mercury substitution of crystalline cytoplasmic actin-DNAase I complex was obtained. Furthermore, no gross structural differences between cytoplasmic and skeletal-muscle actin could be observed at this resolution.

Discussion

Crystals of cytoplasmic actin have been available for some time: actin from bovine spleen in complex with profilin, but without bound nucleotide (Carlsson *et al.*, 1976) and from *Acanthamoeba castellanii* actin in complex with bovine pancreatic DNAase I (Sugino *et al.*, 1979). However, they have been of different space groups, therefore the obtained structural information from these crystals will not be easily comparable with the determined structure of skeletal-muscle actin (Suck *et al.*, 1981). The crystals of porcine liver actin-DNAase I complex obtained have space-group and unit-cell dimensions identical with those of the corresponding crystalline sarcomeric actin-DNAase I complex (Suck *et al.*, 1981). Therefore a direct comparison of the three-dimensional structure of the two actins is feasible by the Fourier-difference procedure.

In cytoplasmic actins the cysteine residue at the *N*-terminus is located at position 17 (Vandekerckhove & Weber, 1978a). In sarcomeric actin this cysteine residue is at position 10, and smooth-muscle actin has cysteine residues at position 10 and 17 (Vandekerckhove & Weber, 1978a,b). Trypsin treatment of actin-DNAase I complex most probably removes a C-terminal tri- or dipeptide (Jacobson & Rosenbusch, 1976; the present work). By analysis of the thiol-group reactivity towards Nbs_2 it has been found that the additional thiol group at the *N*-terminus of smooth-muscle actin (no. 17) is accessible before, but not after, complexing this actin with DNAase I (Mannherz *et al.*, 1983). The results presented here show a large decrease in the number of cysteine residues accessible to Nbs_2 for porcine liver actin after complexing with DNAase I. Also, heavy-atom substitution of crystalline smooth-muscle actin-DNAase I complex with MeHgAc has been possible (Suck *et al.*, 1981), but was not with crystalline porcine liver actin-DNAase I complex. Therefore the most likely position of the mercury atom in crystalline sarcomeric actin-DNAase I complex is cysteine-10 (Suck *et al.*, 1981). The fact that this heavy-atom derivative could not be generated with crystalline cytoplasmic actin-DNAase I complex also strongly supports the notion that the actin employed in this study is of cytoplasmic origin and is not contaminated by smooth-muscle actin, which could have been co-purified after tissue homogenization.

Diffraction data up to 0.6 nm (6 Å) resolution were collected in order to probe for possible structural differences at this resolution by the difference-Fourier procedure. Before data collection, crystals were soaked in 1 mM-MeHgAc for 1.5 h at 4°C in order to achieve heavy-atom substitution as described previously for crystalline skeletal-muscle actin-DNAase I complex (Suck *et al.*, 1981);

Cytoplasmic actins differ from sarcomeric actin by at least 25 amino acid residues (Vandekerckhove & Weber, 1978*a,b*), but all exchanges have been shown to be conservative. Although for porcine liver actin a comparative study of its primary sequence is not available, it can be assumed that the so-far-observed strict tissue specificity of the amino acid sequence of all mammalian actins also pertains to liver actin. No structural differences between the two actins could be detected at low resolution [0.6 nm (6 Å)]. The present study therefore allows us for the first time to extend the observed highly conserved primary structure of actin to the three-dimensional level. The reported functional differences (Gordon *et al.*, 1976*a,b*) between these two actins must be due to more subtle structural changes involving amino acid side chains, which one hopes might be resolved at high resolution. The firm evidence for the mercury atom of the MeHgAc derivative of crystalline skeletal-muscle actin-DNAase I complex being at position 10 will certainly aid the high-resolution analysis of all crystalline actin-DNAase I complexes.

In addition, these results, together with already published evidence (Mannherz *et al.*, 1983), indicate that the contact sites of DNAase I with actin are within the *N*-terminal region of actin, involving a region around residue 17 and the lysine residues at positions 61 and 68, since this region of the *N*-terminal peptide of actin is completely protected against the proteolytic attack of trypsin by DNAase I (the present work; Mannherz *et al.*, 1980, 1983).

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