PHALLOTOXINS BIND TO ACTINS

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1. Introduction

The function of receptors can be ascribed to the filamentous structures which appear with increased frequency in the cytoplasma fraction of rat liver after in vivo or in vitro intoxication with phalloidin [1]. Using [^{3}H]desmethylphalloin we found that 3.6 nmoles of the phallotoxin are tightly bound to 1 mg of filamentous protein [2]. Shortly thereafter it was recognized that the phallotoxin induced filaments from liver (Ph-filaments) bind heavy meromyosin (of rabbit muscle) in an arrow head-like manner like muscle F-actin and so consist of an actin [3]. In contrast to F-actin, however, they are not depolymerized by 0.1 M potassium iodide. Consequently F-actin was also treated with phalloidin and thereupon lost its sensitivity against 0.1 M KI [3]. The modified form, Ph-actin, is formed from G-actin in presence of phalloidin already in KCl free solution provided sufficient Mg^{2+} ions are present. The formation of analogous Ph-actins by several toxic derivatives of phalloidin, and the failure of their formation by nontoxic ones could be stated then by viscosimetry [4]. In the present communication we describe comparative quantitative studies on binding of ^{3}H- and ^{14}C-labelled phallotoxins to Ph-actin obtained by polymerization of G-actin in presence of the toxins and to F-actin (which is transformed into a KI-resistant Ph-actin by the toxins).

G-actin ⟶ Ph-actin
↓ Phallotoxin
F-actin ⟶ Ph-actin

2. Material and methods

2.1. *Reagents*

[^{3}H]Desmethylphalloin was prepared according to ref. [5] and had a specific activity of 32.5 μCi/mg. $N^{(ind)}$-[^{14}C]methylphalloidin was prepared by Dr H. Faulstich according to ref. [6] using [^{14}C]methyliodide. It had a specific activity of 20 μCi/mg. From him was also a sample of (S)-phalloidin sulfoxide. F-actin free from troponin and tropomyosin was a gift of Dr P. Dancker, who had prepared it from rabbit skeletal muscle according to ref. [7]. For obtained G-actin the F-actin pellet was depolymerized in 1 mM Tris–HCl, pH 7.4 by storing for about 3 hr at 20°C in a concentration of 0.68 mg/ml. Radioactivity was measured by digesting 250 μl of the probes with 500 μl of NCS (Tissue-solubilizer-Amersham/Searle)/methylcellosolve/tuluol (2:2:1) at 50°C for 60 min and neutralising the samples with 0.6 N HCl. 12 ml of toluol/methylcellosolve (2:1) scintillator was added and the disintegrations per minute were counted in a Tracerlab Corumatic-200 scintillation counter.

2.2. *Incubations*

Approx. 1 mg each of G-actin or F-actin (in about 2 ml Tris–HCl, pH 7.4, containing 0.7 mM $MgCl_2$) was incubated for 15 min at 37°C with 4 μg of one of the radioactive phallotoxins. Two ml of the solutions were either put on a chromatographic column of Sephadex (see below) or subjected to ultrafiltration.

2.3. *Chromatography*

Chromatography of ^{3}H-labelled Ph-actin was carried out with a column (1.5 × 40 cm) packed with Sephadex G-200 (Pharmacia) in a buffer of pH 8.5

consisting of 0.025 M Tris–HCl, 0.1 M NaCl, 1 mM EDTA and 5.0 mM β-mercaptoethanol.

2.4. *Ultrafiltration*

An Amikon ultrafiltration apparatus of 10 ml volume and 2.5 cm diameter was used with filter UM-10 (Amikon). Two ml of the solution of labelled Ph-actin were diluted to 10 ml with a 0.7 mM solution of $MgCl_2$ and concentrated by pressure (3 atm) to a vol of 2 ml. The dilution and filtration procedure was repeated until the filtrate contained no more radioactivity (5 times). In order to determine the amount of labelled toxin exchangeably by cold toxin the solution free from low molecular radioactivity was incubated with 5 μg of cold phalloidin for 15 min at 37°C and separated from labelled phallotoxin eventually released by ultrafiltration as described above. The exchange procedure was repeated until the radioactivity of the protein solution remained constant. An analogous experiment was carried out using 100 μg of the nontoxic (S)-phalloidin sulfoxide [8].

3. Results and discussion

The transformation of F-actin into KI-resistant Ph-actin is brought about by a strong adsorption of the phallotoxin. This is proved by chromatography. The protein peak which is eluted between 30 ml and 80 ml from an 1.5 X 40 cm column of Sephadex G-200 as registered by U.V. adsorption coincides exactly with radioactive labelling.

A radioactive conjugate of higher molecular weight has already been observed in former chromatographic experiments with homogenates of livers of rats poisoned with [^{3}H]desmethylphalloin [5]. It seems conceivable that this fraction consisted of the analogous Ph-filaments discovered later by electron microscopy [1]. The amounts of phallotoxins bound firmly to F-actin agree very well for both of the labelled toxins, but differ from those bound to G-actin causing its polymerization to Ph-actin. About 6 nmoles of toxins are bound per 1 mg protein, when it is added to the solution of G-actin and the polymerisation forming Ph-actin takes place in presence of a slight excess, (which afterwards is washed out by ultrafiltration). Compared to it only about 1 nmole per 1 mg is fixed to F-actin polymerized from G-actin by 0.1 M KCl (in presence of 0.7 mM $MgCl_2$) transforming it to a less loaded Ph-actin. Therefore it seems adequate to differentiate between the Ph-actins by disignating the higher loaded species Ph-actin G and the low loaded one Ph-actin F.

Table 1
Amounts of labelled phallotoxins bound to Ph-actin formed from rabbit muscle G-actin by the toxins (Ph-actin G) and to Ph-actin likewise formed from F-actin (Ph-actin F)

Phallotoxin	nmoles toxin bound to 1 mg protein (approx. 20 nmoles G-actin)	
	Ph-actin from G-actin (Ph-actin G)	Ph-actin from F-actin (Ph-actin F)
[^{3}H]desmethylphalloin	5.78 6.12	0.94 1.18
N-[^{14}C]methylphalloidin	5.90	0.80

Displacing experiments of labelled phallotoxin by unlabelled phalloidin in both of the Ph-actins gave the results shown in fig. 1. The higher loaded Ph-actin G successively loses the majority of its label by

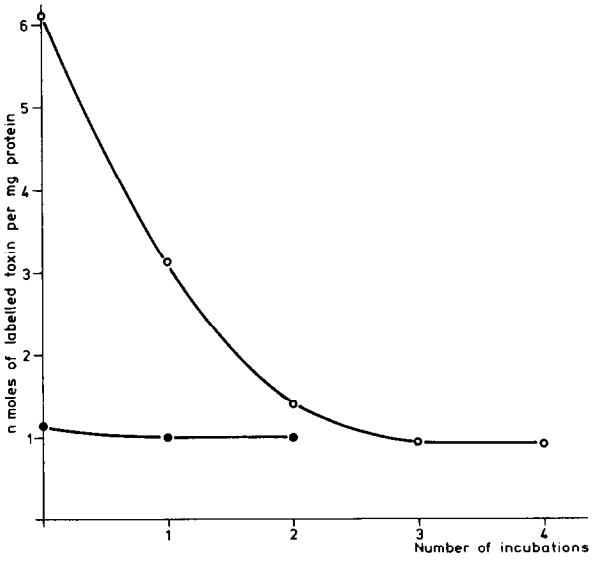

Fig. 1. Decrease of label of Ph-actin G by several successive incubations with unlabelled phalloidin. Amount of labelled phallotoxin bound to Ph-actin during its formation from G-actin (Ph-actin G = ○) and to ready polymerized F-actin (Ph-actin F = ●).

exchange until a level of about 1 nmole of [^{3}H]phallotoxin remains. That is the same amount of phallotoxin which is firmly fixed to Ph-actin F, and which cannot be exchanged by cold phalloidin. In an analogous experiment with two successive incubations with 100 μg of the nontoxic phalloidin sulfoxide [8] the amount of [^{3}H]desmethylphalloin was reduced from 7 nmoles pro mg protein to 6.6 nmoles.

It appears that for transformation of F-actin into the Ph-modification at least one molecule of toxin pro 20 units of G-actin is necessary and indispensable. The excess of toxin incorporated when added to the oligomeric actin (G-actin) is adsorbed less tightly although not easily removable by buffer solution nor by a nontoxic derivative of phalloidin, but is displaced by unlabelled phallotoxin. A ratio of about 1 mol toxin to 6 moles of G-actin (mol. wt. $3 \cdot 10^5$) as found with a preparation strongly enriched in Ph-filaments of liver of a poisoned rat [2] makes it probable that not ready polymerized F-filaments but rather G-actin-like protein is exposed to the action of phallotoxins in the moment of intoxication. The shifting by phallotoxin to the F-actin side (Ph-actin G) in a dynamic equilibrium between G-actin- and F-actin-like proteins situated close to the cytoplasmic membrane seems to give rise to the impairment of the liver cell by the toxins.

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