THE INTERACTION OF PHALLOIDIN, SOME OF ITS DERIVATIVES, AND OF OTHER CYCLIC PEPTIDES WITH MUSCLE ACTIN AS STUDIED BY VISCOSIMETRY

I. LÖW and Th. WIELAND

Max-Planck-Institut für medizinische Forschung, Abteilung Chemie, D-69 Heidelberg, Jahnstr. 29, W. Germany

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1. Introduction

Phalloidin (Ia), a component of the toxic green deathcap toadstool *Amanita phalloides* [1] induces in vivo as well as in vitro the formation of filamentous structures in the cytoplasmic membrane fraction of (rat) liver as observed by electron microscopy [2]. The nature of these filaments as actin has been proven by their reaction with heavy meromyosin to give arrow head-like structures [3]. In contrast to F-actin, however, are the phalloidin-induced fibrils (Ph-filaments) resistant against 0.6 M KI. Consequently also F-actin from rabbit muscle was treated with the toxin and could so be transformed into a modified form (Ph-actin), which proved resistant against 0.6 M KI too. Ph-actin is also formed from G-actin by adding phalloidin to a solution of low ionic strength but sufficiently high ((0.7 mM) Mg^{2+} concentration. Since the process of polymerization of G-actin to F-actin (Ph-actin) can be followed more easily by viscosimetry we studied the interaction of G-actin with phalloidin (Ia), phallacidin (Ib), desmethylphalloin (Ic), its toxic and non-toxic sulfoxides (Id, Ie), the non-toxic derivatives desthiophalloidin (If) and secophalloidin (Ig) as well as α-amanitin and a soluble preparation of antamanide. Further the inhibiting effect of cytochalasin B (CB) on the formation of Ph-actin was investigated.

2. Materials and methods

2.1. *Reagents*

Phalloidin and its derivatives Ib–g and α-amanitin were samples from our laboratory. O-(β-Aminoethyl)-Tyr^6-antamanide was a gift of Dr.Chr. Rietzel. Stock solutions of the cyclic peptides were prepared containing 4 mg of each per ml of water. CB was a sample from the Aldrich Chem. Company. F-actin, free from troponin and tropomyosin as well as myosin was a gift of Dr. P. Dancker, who had prepared it from rabbit skeletal muscle according to ref. [4]. For obtaining G-actin, F-actin pellets were depolymerized in 1 mM Tris–HCl, pH 7.4, by storing for about 3 hr at + 4°C at a concentration of 1.2 mg/ml. Only very few filaments were left over as checked by electron microscopy [3]. The solution of myosin in 0.5 M KCl contained 3 mg/ml. CB was applied as a solution in dimethylformamide containing 20 mg/ml. Other solutions used were: ATP–Na, pH 7.4, 50 mM; EDTA-Na, pH 7.4, 0.1 M; EGTA-Na, pH 7.4, 20 mM; $MgCl_2$, 0.15 M; *N*-ethylmaleimide, 0.1 M. KCl and KI were added as solids.

2.2. *Determination of viscosity*

The viscosity of 2 ml samples was assayed in a spiral capillary viscosimeter, in which the flow time between two marks was 30 sec for water and about the same for G-actin (1.2 mg/ml) in 1 mM Tris buffer. Reagents were added with micropipettes directly into the viscosimeter; solid KI and KCl were dissolved outside. Protein was determined by the method of Lowry et al. [5].

2.3. *Incubation of G-actin with the reagents*

Two ml samples (2.4 mg) of the G-actin solutions were incubated with 20 μl (80 μg = 2 moles per mole actin) of the peptide solutions either overnight at

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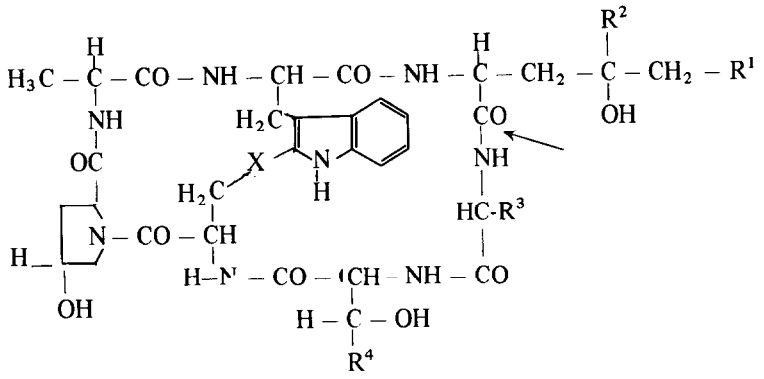

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No.	Compound	R^1	R^2	R^3	R^4	X	Tox.	Ref.
a	Phalloidin	OH	CH_2OH	CH_3	CH_3	S	+	[1]
b	Phallacidin	OH	CH_2OH	$CH(CH_3)_2$	CO_2H	S	+	[1]
c	Desmethylphalloin	H	H	CH_3	CH_3	S	+	[1]
d	Phalloidinsulfoxide B	OH	CH_2OH	CH_3	CH_3	S=O (R-conf.)	+	[6]
e	Phalloidinsulfoxide A	OH	CH_2OH	CH_3	CH_3	O=S (S-conf.)	–	[6]
f	Desthiophalloidin	OH	CH_2OH	CH_3	CH_3	–H H–	–	[1]
g	Secophalloidin	OH	CH_2OH	CH_3	CH_3	S (peptide bond [arrow] hydrolyzed)	–	[1]

Formulae I (a–g).

+4°C or for 30 min at room temperature. Ten to twenty microlitres of $MgCl_2$ solution were added to guarantee the proper conditions, although the solution might already have contained sufficient Mg^{2+} per se. CB was applied by adding 20 μl of the DMF solution and incubated for 15 min at 30°C prior to the addition of phalloidin (80 μg). The soluble antamanide (0.1 ml = 400 μg) was incubated with the solution of G-actin 30 min at 21°C, then 20 μl of the phalloidin solution was added. In the case of F-actin formation the viscosity rose so as to produce a flow time of about 60 sec and dropped to 30 sec shortly after addition of 200 mg KI; no change of high viscosity indicated the presence of Ph-actin.

3. Results and discussion

The results of our studies on the formation of filamentous actin in the presence of several cyclic peptides have been summarized in table 1. No polymerization of G-actin occurs in a solution of low ionic strength unless sufficient Mg^{2+} ions are present. In the absence of KCl, however, 0.7 mM $MgCl_2$ also brings about only partial polymerisation as compared with 0.1 M KCl. The F-actin formed under these conditions is totally depolymerized by 0.6 M potassium iodide. Phalloidin induces polymerization of G-actin also in ion free medium, the Ph-actin formed by this drug being resistant against 0.6 M potassium iodide. This confirms our electron optical observations made on phalloidin-induced filaments (Ph-filaments) in rat liver and in muscle actin (Ph-actin [3]). The equally toxic phallacidin (1b) and desmethylphalloin (Ic) also convert G-actin into a 'phallotoxinized' actin, which withstands the depolymerizing action of 0.6 M KI. Two diastereomeric sulfoxides, Id and Ie, have been obtained by oxidation of Ia [6] of which only

Table 1
Flow times (viscosities) of solutions of G-actin (1.2 mg/ml 1 mM Tris–HCl, pH 7.4 + 0.7–1.0 mM Mg^{2+}) after incubation with KCl or different toxic and nontoxic cyclic peptides

Compound added	Flow time (sec) at 21°C	
	30 min after addition	After subsequent addition of 200 mg KI
None, without additional Mg^{2+}	32	–
None, (0.7 mM Mg^{2+})	45	28
KCl (0.1 M)	62–65	28
Phalloidin (1a)	63–66	58
1 mM EDTA, KCl (0.1 M)	30	–
1 mM EDTA, phalloidin	30	–
0.2 mM EGTA, KCl	~ 62	30
0.2 mM EGTA, phalloidin	~ 62	55
KCl without additional Mg^{2+}	60	28
Phalloidin without additional Mg^{2+}	< 60–30	–
Phallacidin (Ib)	60	50
Desmethylphalloin (Ic)	66	58
Phalloidinsulfoxide B (Id)	63	58
Phalloidinsulfoxide A (Ie)	60	30
Desthiophalloidin (If)	60	30
Secophalloidin (Ig)	60	30
α-Amanitin	60	30
Soluble antamanide	55	29
KCl after pretreatment with soluble antamanide	60	30
Phalloidin after pretreatment with soluble antamanide	60	54
Phalloidin after pretreatment with CB	30	–

Id showed toxicity. In accordance only the toxic Id induced the formation of KI-resistant filaments. Phalloidin brings about formation of Ph-actin, although very slowly (3–4 hr), from G-actin even in the presence of 0.6 M KI, if adenosine triphosphate was added in advance. The non-toxic Ie, it is true, led also to an increase of viscosity, which, however, was reversed after addition of KI, thus showing that no phallotoxin-like interaction of Ie with actin had occurred, but F-actin has been formed. The same was true also with the non-toxic derivatives of phalloidin If and Ig, with α-amanitin and the soluble antamanide. All of them induced the formation of F-actin in an ion-depleted medium, providing Mg^{2+} ions were present in sufficient high amounts. The soluble antamanide did not antagonize the effect of phallodin in accordance with results of former investigations [7]. The repression of filament formation in rat liver cytoplasmic membranes by in vitro intoxication with phalloidin after pretreatment with antamanide and observed by electron microscopy [2] is being further investigated. CB prevented the polymerization of G-actin to Ph-actin as it also prevented Ph-filament formation in rat liver [8].

The transformation of F-actin to Ph-actin can also be achieved in the polymerized state: F-actin filaments are converted to Ph-actin filaments, resistant against 0.6 M KI, by incubation with phalloidin. This reaction occurs also without Mg^{2+} ions, with 1 mM EDTA present. The reaction with phalloidin does not depend on SH-groups; after treatment of F-action with *N*-ethylmaleimide (1 mM) phalloidin still exerted its stabilizing effect. Ph-actin was also stable against 1 mM ATP-Na in ion-free medium.

We also examined the *coupling reaction* of F-actin and of Ph-actin with myosin in the presence of phalloidin. To 2.0 ml of F-actin and 2 ml of Ph-actin in 0.5 M KCl solution 0.2 ml of the myosin solution

(0.6 mg) was added and immediately mixed. The flow time of the F-actomyosin sample was 163 sec and dropped to 115 sec after standing for 22 hr. Ph-actin also coupled with myosin; the Ph-actomyosin so formed effected a flow time the same as F-actomyosin (160 sec), which, however, did not fall within 22 hr. Thus the actomyosin complex also seems to be stabilized by the toxin. In 0.6 M KI solution, however, complete dissociation into Ph-actin (flow time 60 sec) and myosin occurred within 20 hr.

The viscosimetric investigations confirmed our results obtained by electron microscopy [3] and over that showed the possiblity of proving in a simple way, whether derivatives, analogues or synthetic bicyclic peptides have phalloidin-like toxicity. It is most probable that the toxic action on liver cell membranes and the stabilizing effect of phalloidin on polymeric actin has a common source. The polymerization of G-actin, also in the absence of the ionic strength normally necessary, may be best explained by assuming that the monomeric G-actin reacts with phallotoxins in such an (allosteric) manner, that it can no more dissociate from the dimeric, trimeric, etc. up to the polymeric state, and so the equilibrium G-actin $\rightleftharpoons$ F-actin, perhaps indispensable for normal functioning of the membrane, is shifted totally and irreversibly to the polymer Ph-actin state.

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