RECIPROCAL INTERACTIONS OF RICIN FROM *RICINUS COMMUNIS* L. SEEDS WITH EUKARYOTE RIBOSOMES

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1. Introduction

Ricin, the phytotoxin of castor bean seeds, is highly toxic for animals and has been isolated in a pure state [1,2]. It is biochemically, physiologically, and toxicologically different [3] from the two lectins, which show a hemagglutinating activity [4,5], isolated from these seeds. The site of ricin-action was first established by Dirheimer et al. [6], who showed that ricin inhibits eukaryote protein synthesis at the ribosomal level [6]. Later it was shown that this toxin inhibits protein synthesis in intact cells [7] as well as in cell-free systems [8], but only in eukaryotes [9,10]. However, we found that ricin inhibits fungal mitochondrial protein synthesis [10], though Greco et al. [11] could not find such an inhibition in rat liver mitochondria.

The mechanism of ricin-action on protein synthesis at the ribosomal level is currently a source of controversy. The toxin acts on the 60 S ribosomal subunit [12], blocking the elongation step [8] of protein synthesis by inhibiting the binding of elongation factor 2 (EF 2) to the 80 S ribosome, but without acting on EF 2 itself [13–17]. Ricin has no effect on the peptide-bond formation [13,14,16] but is reported to be an inhibitor of translocation by some authors [15–18], but not by others [19,20]. The elongation factor 1-dependent GTPase activity of ribosomes is inhibited by ricin [16,18–20], but the EF 1-dependent binding of aminoacyl-tRNA to ribosomes is scarcely or not at all affected by ricin, according to several authors [16–18], whereas others find that it is strongly inhibited [14]. Finally it has been reported recently that inhibition of protein synthesis by ricin depends strongly upon the concentrations of ribosomes and elongation factors EF 2 and EF 1: at saturating concentrations they prevent ricin-action [20].

In this paper we show that ricin is inhibitory only during active protein synthesis, because it is only in this phase that ricin is cleaved into its subunits, one of them being the active, inhibitory form. In these conditions the inactivated polysomes are stabilized against salt-dissociation. A preliminary report of these results was already given [21].

2. Materials and methods

2.1. *Materials*

The seeds of *Ricinus communis* L. were kindly supplied by 'Société Française du Ricin', 20 Rue Grignan, 13001 Marseille, France. ATP, GTP, creatine phosphate, creatine phosphokinase, non-labeled amino acids and hemin were purchased from 'Sigma', St. Louis, Mo., USA. L-[^{14}C]Valine (140 Ci/mol) was from the 'Commissariat à l'Energie Atomique', Saclay, France and Na^{125}I (10 mCi/μg) was obtained from the 'Radiochemical Centre', Amersham, UK. Omnifluor was from 'NEN Chemicals

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GmbH', Dreieichenhain, FRG and NCS tissue solubilizer came from 'Amersham/Searle Corporation', USA. All chemicals used were the purest available from 'Merck', FRG, 'Fluka', Switzerland or 'Boehringer Mannheim', FRG.

2.2. *Ricin preparation*

Ricin was prepared as previously described [2]. Concentrations of ricin were determined according to Lowry et al. [22], using human serum albumin as standard.

2.3. *Ricin labeling*

Labeled ricin with four radio-iodine atoms/molecule (spec. act. 16×10^7 cpm/μg) was prepared as previously described [23], so that each of the two A- and B-chains was uniformly labeled (2 radio-iodine atoms/chain) and fully active.

2.4. *Eukaryotic cell-free protein synthesis*

The eukaryotic cell-free protein synthesis system was prepared from rabbit reticulocytes, as described earlier [24]. The incubation mixture (1 ml) contained: 0.40 ml reticulocyte lysate, 75 mM KCl, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 40 units creatine phosphokinase, 15 μM hemin, 1 μCi [^{14}C]valine and 0.02 mM each of the other 19 non-labeled amino acids. The incubations were carried out at 27°C for various times. Afterwards 50 μl samples were put onto Whatman 3 MM paper discs which were treated and counted as previously described [24]. In none of these steps was 2-mercaptoethanol or another reducing agent used.

2.5. *Polyacrylamide gel electrophoresis*

After different periods of incubation 100 μl portions of the cell-free system were layered onto 7.5% polyacrylamide gel cylinders and submitted to electrophoresis according to Davis [25], at pH 8.3. At the end of the electrophoresis the gels were quickly removed from the tubes, frozen at −80°C, and uniformly sliced with a mechanical fractionator into pieces 1.5 mm thick. Each gel-piece was put in a plastic vial and the radioactivity of ^{125}I-labeled protein was measured in a Packard auto-gamma scintillation spectrometer. The small interference from beta-radioactivity was automatically subtracted.

2.6. *Density-gradient analysis of polysomes*

After different times of incubation, the reticulocyte cell-free system was layered over a 34 ml linear sucrose-gradient (10–40%, w/v) in 5 mM $MgCl_2$, 25 mM NaCl, 25 mM Tris–HCl buffer, pH 7.5, and was centrifuged at 26 000 rev./min in the SW27 rotor of a Spinco Model L ultracentrifuge for 2.5 h at 4°C. The absorbance at 254 nm was automatically determined with an Isco gradient-spectrometer during the collection of the gradients.

3. Results

3.1. *Pre-protein inhibition lag-time study*

When different ricin-concentrations were added to rabbit reticulocyte protein-synthesizing system, percentage inhibition of protein synthesis increased with increasing concentrations of toxin, but 100% inhibition was never reached even with high ricin-doses (fig.1).

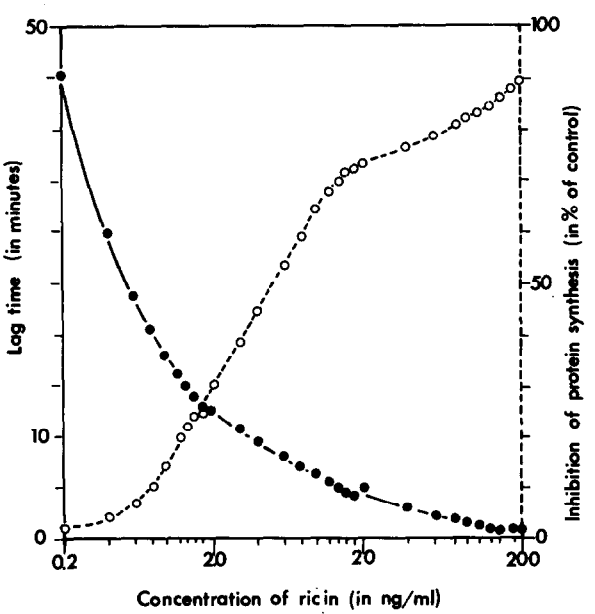

Fig.1. Lag-time of protein synthesis inhibition as a function of ricin concentration in rabbit reticulocytes lysate. (o- - -o) Inhibition of protein synthesis (in %), (•——•) lag time (in min). These values were determined from kinetics of [^{14}C]valine-incorporation according to Materials and methods. The percentage inhibition was measured after 30 min incubation.

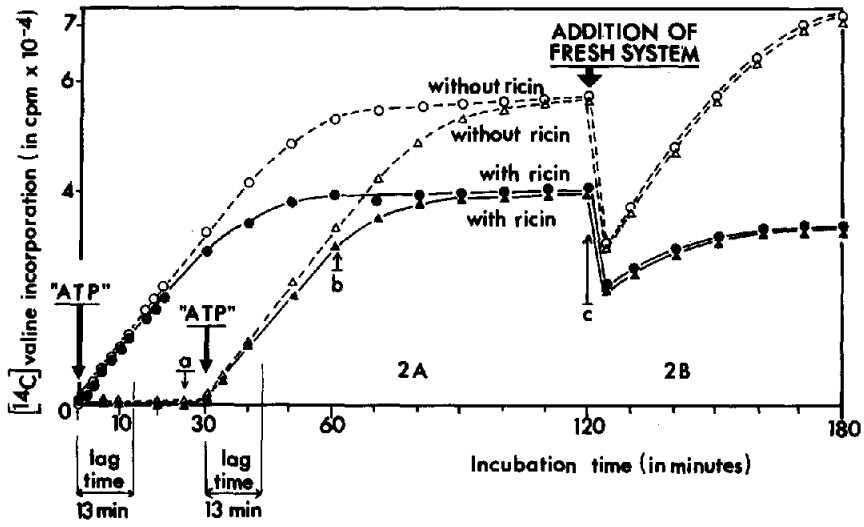

Fig.2A. Kinetics of protein synthesis in rabbit reticulocyte cell-free system at 27°C, in the presence (—●— and —▲—) or absence (- - -○- - - and - - -△- - -) of ricin, without (- - -○- - - and —●—) or with (- - -△- - - and —▲—) a preincubation for 30 min without, an energy source. ATP stands for the energy source, i.e., ATP and its regenerating system creatine phosphate and creatine phosphokinase. Ricin concentration: 2 ng/ml. For arrows (a), (b) and (c), see legend of fig.3. Fig.2B. At 120 min the residual volume of the reaction mixtures of fig.2A (250 μl) was completed with 250 μl fresh cell-free system with energy source and without ricin. Then protein synthesis was measured after different times of incubation. The fall in radioactivity at the beginning of this experiment was due to the dilution of the reaction mixtures in the ratio 1:1.

3.2. *Protein synthesis kinetics with or without energy source*

Figure 2A shows the protein synthesis kinetics in absence or presence of 2 ng/ml ricin. Without an energy source (ATP, creatine phosphate and creatine phosphokinase) no synthesis occurred. When the energy source was added, after 30 min in this experiment (fig.2A), percentage inhibition of protein synthesis and the lag-time were identical (30% and 13 min, respectively) with those obtained in the corresponding experiment where ricin was added immediately to complete cell-free system (fig.2A).

3.3. *Protein synthesis kinetics after new cell-free system addition*

Figure 2B shows protein synthesis kinetics after addition of a fresh cell-free system (with energy source but without ricin). In these conditions the lag-time disappeared and protein synthesis was more inhibited than in experiments shown in fig.2A. Indeed, 60 min after addition of fresh-system, protein synthesis was 75% inhibited, whereas it had been 30% inhibited in the experiments shown in fig.2A. Furthermore it must be emphasized that in the experiment shown in fig.2B the ricin concentration was half that in the experiment shown in fig.2A, due to dilution with fresh-system. In conclusion, the inhibition is 5-times greater with ricin preincubated in presence of protein synthesizing system than with native-ricin.

3.4. *Labeled-ricin electrophoretic patterns in protein synthesis experiments*

The preceding experiments were repeated with ^{125}I-labeled ricin. Samples were taken from the reaction mixtures and submitted to polyacrylamide gel electrophoresis, to examine ricin integrity. Ricin incubated for 30 min in the protein synthesizing system, without energy source, migrated as a single peak at the position of native ricin (fig.3). On the other hand ricin, after 30 min or 60 min incubation, in protein synthesizing system with an energy source, was almost completely cleaved into two peaks. They migrated at the same positions as the two polypeptide chains, A and B, which can be obtained by 2-mercaptoethanol treatment of ricin [26].

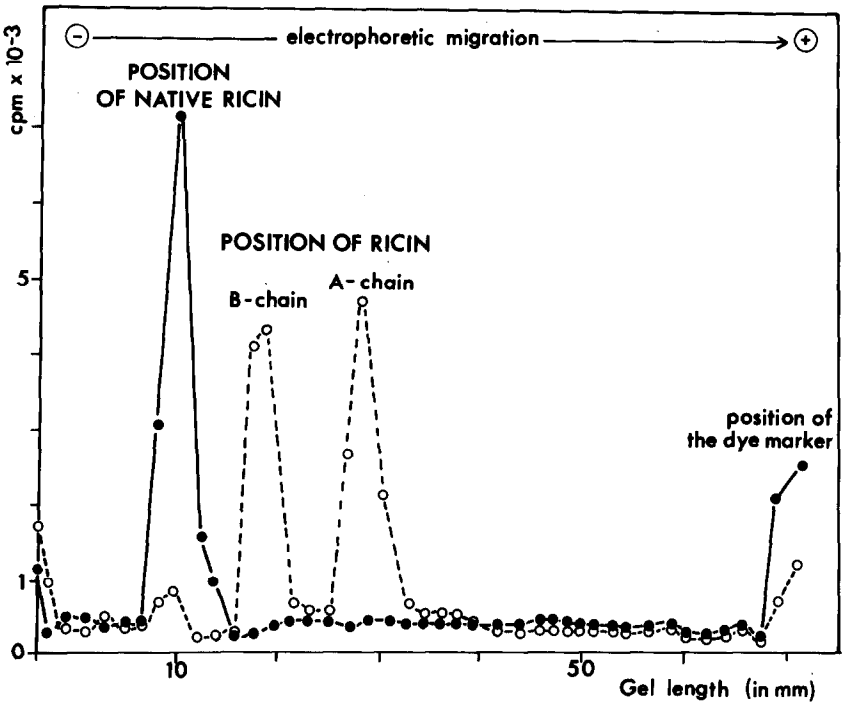

Fig.3. Electrophoretic patterns of tetraiodo-labeled ricin, after different times of incubation with a protein-synthesizing system from rabbit reticulocytes. The incubation system was the same as in fig.2 but with 2 ng ^{125}I-labeled ricin/ml cell-free system. After 25 min incubation in absence of the energy source (arrow 'a' fig.2A) and after 30 min and 60 min of incubation, in the presence of ATP and its regenerating system (arrows 'b' and 'c' fig.2A) samples were submitted to electrophoresis. (•——•) Corresponds to 'a', (o- -o) corresponds to 'b' or 'c'. About 15% total radioactivity remained on the spacer-gel due to some aggregation of the molecules on the gel, and about 8% radioactivity was found with the dye-marker due to some radiolysis during electrophoresis.

3.5. *Ricin-action on rabbit reticulocyte polysomes*

The rabbit reticulocyte cell-free protein synthesizing system normally containing 75 mM KCl was incubated with a high dose of ricin (ricin/200 ng/ml reaction mixture, which caused 90% inhibition and a lag-time of less than 1 min) for various times (0 min, 30 min and 60 min) at 27°C. Sedimentation profiles of polysomes were analyzed (according to Materials and methods) for each incubation time. In each experiment controls had no ricin. Figure 4A shows that the profiles of the polysomes obtained from ricin-treated reticulocyte reaction mixtures at different times were identical with control, carried out at 0 min, although protein synthesis was 92% inhibited by ricin after 30 min of incubation.

Figure 4B shows that in the presence of a high concentration of salt (200 mM KCl), in the cell-free system, the control polysomes were disaggregated to monosomes, whereas the polysomes of the ricin-treated system remained mostly preserved.

4. Discussion

Ricin is a glycoprotein composed of two chains [26]. The heavier chain, called Ala-chain [26] or B-chain [27], binds to the membrane of mammalian cells [27] and the lighter chain, called Ile-chain [26] or A-chain [27], contains all the toxicity of the molecule [27] and specifically blocks protein synthesis [13,27]. It was shown [9–20,27] that ricin and its A-chain irreversibly inactivate the 60 S ribosomal subunit and strongly reduce its binding with EF 2. However, some important problems concerning this mode of action by ricin remain to be investigated [20].

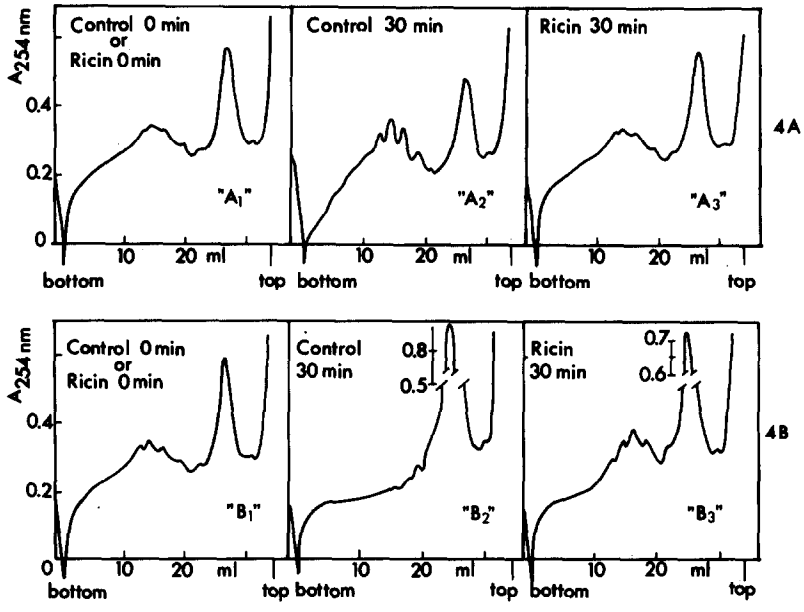

Fig.4. Polysome sedimentation profiles from controls or a ricin-treated reticulocyte reaction mixture in the presence of 75 mM KCl (normal system 'A') or 200 mM KCl ('B'). The concentration of ricin was 200 ng/ml. 'A1' and 'B1': Controls at 0 min with or without ricin. 'A2' and 'B2': Controls without ricin after 30 min incubation. 'A3' and 'B3': Controls with ricin after 30 min incubation.

One of these problems is the lag-time before inhibition of protein synthesis, not only when ricin is incubated with cells [27–29], but also when ricin is incubated in a cell-free system [8,24]. The results presented in this paper clearly show, that ricin blocks protein synthesis always after a lag-time, that decreases with increasing concentrations of the toxin, but is never equal to zero, even with high doses of ricin. This explains why complete inhibition of protein synthesis is never reached, because a certain extent of protein synthesis takes place during the lag-period.

Trying to determine the reason for this lag-time we first supposed that intact ricin degraded some component essential for protein synthesis and we incubated ricin with the complete cell-free system in the absence of an energy source (fig.2A). But after this preincubation, when energy source was added, the protein synthesis worked as well as in the non-preincubated system and was only inhibited after a lag-time identical to that found in the control. This result shows that intact ricin is unable to act on the translation machinery [21].

The lag-time disappeared when ricin had already been incubated with a protein-synthesizing system in the presence of an energy source (fig.2B). This result suggests that ricin must be activated by a working protein-synthesizing system, the lag-time being essentially due to the time necessary for the activation of ricin molecules [21].

To show more directly which process occurred during the activation of ricin by the cell-free system during protein synthesis, we utilized highly-labeled ricin and analyzed, on polyacrylamide gels, samples of the reaction mixture after different incubation times. In these conditions we showed that native ricin is more than 90% split into its A- and B-chains, 30 min after the addition of the energy source. Such a splitting has also been shown by Olsnes et al. [28]. On the other hand, ricin remained intact when incubation was carried out without an energy source.

The polysomes of protein-synthesizing system were also studied after different incubation times in the absence or presence of ricin. The controls revealed that, at 0 min of protein synthesis, there were mostly polysomes, with only a few monosomes.

During incubation, polysomes gradually disaggregated into tetra-, tri-, di- and monosomes. Upon the action of ricin the polysome structure was preserved and was identical to control at 0 min of incubation. This is in accordance with the fact that ricin blocks the elongation of the polypeptide chain [8]. However, our results are discrepant with those of some authors [30–32] who found that ricin acts on the initiation step. It must be pointed out that some of these authors showed that this effect on polysome breakdown in intact cells by ricin is not very strong [32], or even quite small [30], suggesting that ricin may act essentially similarly in cells and in cell-free systems.

It is known that high concentrations of salts, particularly KCl, disaggregate eukaryotic polysomes. Indeed when our control polysomes were incubated with 200 mM KCl the polysome structure was degraded (fig.4B), whereas polysomes of the ricin-treated system were mostly preserved. This means that polysomes inactivated by ricin were 'frozen' and stabilized even against high salt concentrations. The nature of this stabilization is at present unknown.

The reciprocal interaction of ricin with the ribosomes deserves further investigation, to show which ribosomal component, accessible or active only during running protein synthesis, is responsible for the cleavage of ricin into its two subunits, and to find out which component is inactivated by ricin, preventing ribosomes interaction with elongation factor(s) and their dissociation from the messenger RNA.

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