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THE ACTION OF RICIN A-CHAIN ON RIBOSOMES

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The mechanism of action of ricin, an irreversible inhibitor of protein synthesis in eukaryotic ribosomes, has been studied with ethanol extracted 80S rat liver ribosomes. The results show that the irreversible action of the toxin is on a component of the ribosome which remains in the core ribosome after the removal of the ethanol soluble proteins. The acid phosphoproteins P1 and P2 are shown not to be the site of action of the toxin.

INTRODUCTION

Ricin, a toxic protein isolated from the seeds of Ricinus communis beans, is a powerful inhibitor of protein synthesis in eukaryotic systems (1-3). In vitro the toxin induces an irreversible modification of ribosomes which renders them unable to support polypeptide chain elongation. Sperti et al. (4) have shown that elongation factor-2 (EF-2) dependent GTPase activity is inhibited by ricin A-chain while Montanaro et al. (3) have shown that peptidyl transferase and aminoacyl tRNA binding of ricin inactivated ribosomes is left intact. These same investigators have shown that the binding of elongation factor-2 and adenosine diphosphoribosyl-EF-2 is also inhibited by ricin (5). The existing data suggest, therefore, that ricin acts on a component of the ribosome which is essential for the translocation step of protein elongation. The action of ricin as a nuclease has been investigated by Mitchell et al. (6), as a protease by Lugnier et al. (7), and as a phosphatase or kinase by Houston (8). These efforts have not shown a specific enzymatic activity associated with the toxin.

Recently we have identified proteins in the 60S subunit of rat liver ribosomes (phosphoproteins P1 and P2) which are essential for the interaction of ribosomes with elongation factor-2 (9,10). We, therefore, investigated the possibility that P1 and P2 are the site of action of ricin on the rat liver ribosome.

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MATERIALS AND METHODS

[^{3}H]-phenylalanine and Aquasol were obtained from New England Nuclear. Puromycin hydrochloride, sodium deoxycholate, dithiothreotol, and polyuridylic acid were purchased from Sigma Chemical Company. E. coli paste was obtained from Grand Island Biological.

Puromycin treated 80S rat liver ribosomes were prepared according to Silve and Moldave (11). pH 5.2 supernatant containing impure elongation factors 1 and 2 was prepared as described by Moldave and Skogerson (12). Elongation factor-2 was purified according to the procedure of Moldave et al. (13).

Whole ricin and ricin A-chain were prepared from R. communis beans as previously described (14).

Rat liver ribosomes in buffer A (0.35 M sucrose, 0.05 M Tris·HCl, 0.05 M KCl, 0.004 M $MgCl_2$, 0.001 M DTT) were extracted by adding one volume of cold ethanol and a solution of KCl to make the final concentration of salt: 0.025 M Tris·HCl, 0.075 M KCl, 0.002 M $MgCl_2$. This solution was allowed to stand in ice with occasional stirring for 2 minutes then centrifuged at 20,000 X g for 10 minutes. The pellet was resuspended in buffer A for a second extraction and the combined supernatants were dialyzed against buffer A overnight. The resulting ribosomal pellet was also dialyzed in buffer A overnight and then centrifuged at 10,000 X g before reconstituting with the dialyzed supernatants.

Reconstitution was accomplished by adding an appropriate amount of dialyzed extracted protein together with the core ribosome and incubating the two with buffer at 37° C for 15 minutes before assaying.

Rabbit antisera against ricin A-chain was prepared as previously described (10).

RESULTS

Inhibition of Ricin A-Chain Action by Anti-Ricin A-Chain Antibodies

We have prepared rabbit antiserum against ricin A-chain. The action of the IgG fraction from this antiserum was tested by incubating identical amounts of ribosomes in separate tubes with limiting quantities of ricin A-chain for various periods of time, terminated by the addition of an excess of the toxin antiserum. The results shown in Figure 1 clearly demonstrate that the antiserum was capable of stopping the action of ricin A-chain on ribosomes in situ. They also indicate that the action of ricin A-chain is irreversible since the antibody was not capable of reactivating ribosomes which had already been partially or completely inactivated by the toxin.

Extraction and Reconstitution of Ricin A-Chain Treated and Control Ribosomes

Separate ethanol extractions were carried out on ribosomes which had been inactivated with a catalytic amount of ricin A-chain (1:1000 mole ratio of ricin to ribosomes) and ribosomes which were identically treated but without toxin. These extractions yielded two types of 80S core ribosomes and two types of ethanol extracts: 80S core ribosomes resulting from ricin A-chain treated

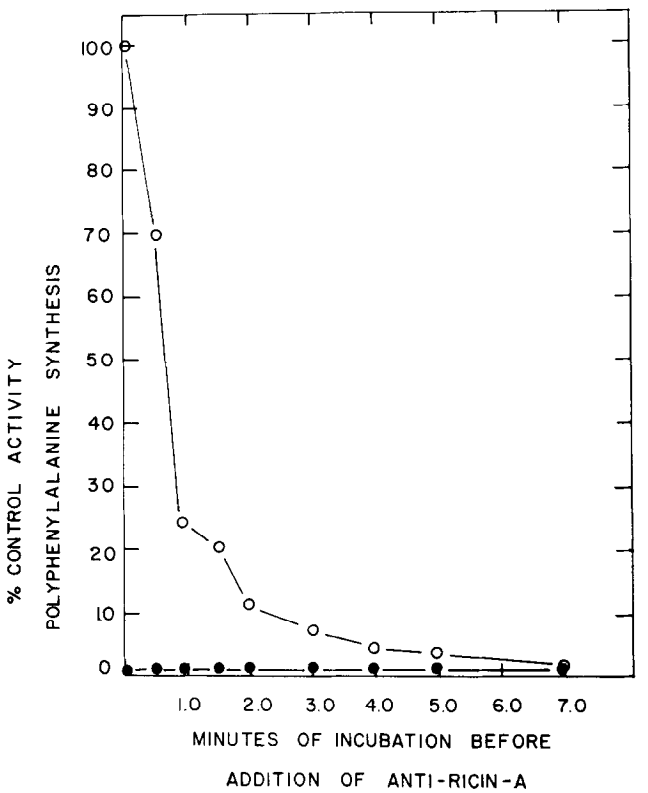

Figure 1. Anti-Ricin A-Chain Antibody Action of Ricin A-Chain Inhibition of 80S Ribosome.

The ability of ricin A-chain antibody to stop the action of ricin A-chain on 80S ribosomes was demonstrated by incubating identical tubes containing 9.0 picomoles of 80S ribosomes and 2.0 nanograms of ricin A-chain for 10 minutes at 37° C. To individual tubes at time intervals of one minute or less a 5.0 microliter solution of ricin A-chain antibody was added and the tube allowed to continue through the preincubation period (o—o). Control activity was determined with ribosomes which had antibody added before the toxin was added. Assays to which no antibody was added are shown (•—•).

ribosomes (RA-80S core), 80S core ribosomes from untreated ribosomes (control 80S core), ethanol extract from ricin A-chain treated ribosomes (RA-ethanol extract), and ethanol extract from untreated ribosomes (control-ethanol extract). The two types of 80S cores and ethanol extracts were separately dialyzed and reconstituted in all possible combinations. Elongation activities of these combinations of reconstituted ribosomes were assayed. To ensure that residual ricin A-chain remaining in the ribosome preparation after extraction did not interfere with the activity of the reconstituted ribosomes, an appropriate amount of anti-ricin A-chain IgG fraction was added to all assay trials before the reconstitution was carried out.

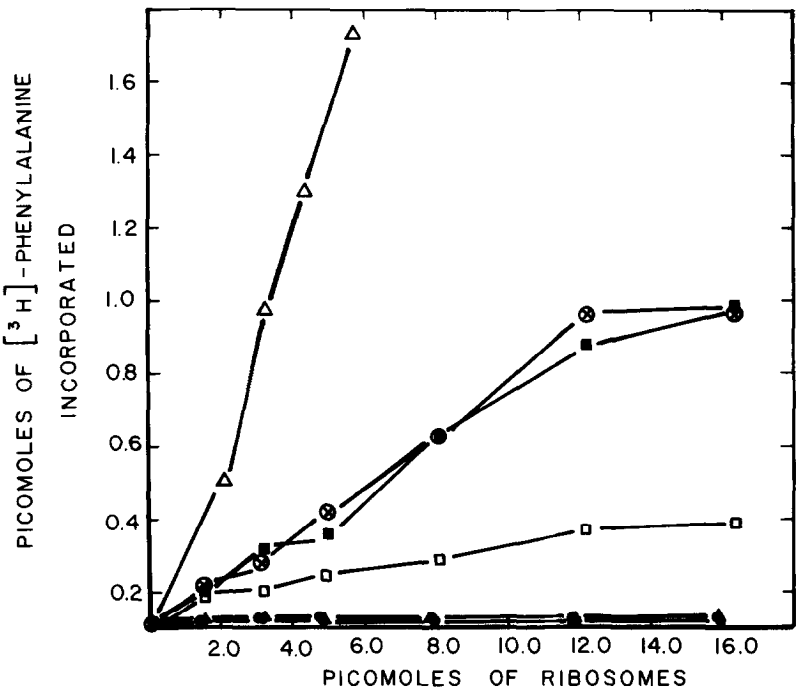

Figure 2. Reconstitution of 80S Core Ribosome with Extracts Obtained from Ricin A-Chain Treated and Control Ribosomes.

80S core ribosomes obtained from ricin A-chain treated and control ribosomes were reconstituted with extracts from the same two types of ribosomes in the following combinations and assayed for polyphenylalanine synthesis activity. Control 80S core ribosomes were reconstituted with extracts from control ribosomes (■), 80S core ribosomes obtained from untreated 80S ribosomes were reconstituted with extracts from ricin A-chain treated 80S ribosomes (⊗), 80S core ribosomes obtained from ricin A-chain treated 80S ribosome were reconstituted with extracts from untreated ribosomes (●), 80S core ribosomes obtained from ricin A-chain treated ribosomes were reconstituted with extracts from ricin A-chain treated ribosomes (▲). The activity of 80S core ribosomes obtained from untreated 80S ribosomes (□) and the activity of 80S core ribosomes obtained from ricin A-chain treated ribosomes (○) were determined for reference along with the activity of unextracted 80S ribosomes (△). The points under the line plotted as (○) are not visible because they fall exactly on top of (▲) and (●).

The results of the poly U directed polyphenylalanine synthesis assays on the different combinations of cross-reconstitution of 80S and 80S core ribosomes with the ethanol extracted protein are shown in Figure 2. Control experiments were included in which unextracted 80S ribosomes were preincubated with control and RA-ethanol extract to demonstrate that the RA-extract did not contain residual ricin A-chain activity in a sufficient amount so as to have had any inhibitory effect on 80S or 80S core ribosomes. Assays of the various ribosome activities were carried out using limiting quantities of ribosomes measured at increasing concentrations holding all other components constant.

Reconstitution of control 80S cores with either RA-ethanol extract or control extract in the presence of anti-ricin A-chain yielded ribosomes with nearly identical elongation activity. RA-80S cores reconstituted with either control or RA-ethanol extract in the presence of anti-ricin A-chain were inactive in polypeptide elongation. Preincubation of unextracted 80S ribosomes with either control or RA-ethanol extract did not change their activity (data not shown). Unextracted 80S ribosomes which had been treated with catalytic amounts of ricin A-chain showed no activity when assayed with or without anti-ricin A-chain IgG fraction. Results identical to those shown in Figure 2 could be obtained without the use of anti-ricin A-chain antibody.

DISCUSSION

The catalytic inactivation of 80S ribosomes by ricin A-chain can be stopped while in progress but not reversed by the addition of anti-ricin A-chain antibodies, consistent with the data of Fodstad and Olsnes (15).

Polyphenylalanine synthesis activity measurements on extracted and reconstituted 80S ribosomes have been used to determine if ethanol extraction removes the site of action of ricin A-chain from the ribosome or if it remains with the 80S core ribosome after the extraction. The data demonstrates that the site of action of ricin A-chain remains with the 80S core ribosome and that acidic phosphoproteins P1 and P2, which we had previously shown to be the active constituents of the ethanol extract (9,10), are not the site of action of ricin A-chain. This conclusion was not expected since the acidic phosphoproteins have been shown to be essential for the same reactions of the elongation cycle (EF-2 GTPase activity and adenosine diphosphoribosyl-EF-2 binding), which ricin A-chain catalytically inhibits (4,5,9,10). The fact that ricin acts on a component of the ribosome which is not removed by ethanol extraction combined with the fact that ricin inhibits steps of the elongation cycle which involve EF-2 allows the conclusion that other component(s) in the ribosome in addition to the acidic phosphoproteins are necessary for the reactions of EF-2. The identification of these components may well lead to the understanding of the mechanism of action of the toxin. This

work represents the first attempt to study the action of a eukaryotic ribosome inhibitor by using partial reconstitution techniques.

ACKNOWLEDGMENTS

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