Vol. 109, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 16, 1982 Pages 7-13

DISUCCINIMIDYL SUBERATE CROSS-LINKED RICIN DOES NOT INHIBIT CELL-FREE PROTEIN SYNTHESIS

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Received September 13, 1982

Ricin was reacted with disuccinimidyl suberate, to yield a molecule in which the A and B chains were covalently cross-linked through a non-reducible bond. After purification, this cross-linked ricin analog was unable to inhibit protein synthesis in a cell-free translation system from rat liver. In contrast, after modification with the cross-linking agent the isolated ricin A chain maintained its inhibitory activity. These results support the view that ricin must be cleaved into its constituent polypeptide chains to elicit its toxicity, and suggest that reduction of the disulfide bond alone is not sufficient for ribosome inactivation in vitro.

Ricin consists of two polypeptide chains linked by a disulfide bond. The B chain binds to galactose-containing receptors on the cell surface (1). The A chain catalytically inactivates the 60S subunit of eukaryotic ribosomes (2,3). While the A chain requires the B chain to inhibit protein synthesis in intact cells (4), only the A chain is necessary for the inactivation of the 60S subunit in vitro (5). Indeed, the A chain may need to be liberated from the B chain to express its toxicity (5). When the disulfide bridge of ricin was substituted by a non-reducible bond, using N,N'-o-phenylenedimaleimide, the ricin analog was incorporated into cells in the same manner as native ricin but was much less toxic, suggesting that reduction of the disulfide bridge is important in eliciting ricin toxicity (6). The kinetics of protein synthesis inhibition in a cell-free system (7) also support the view that the two chains must separate for expression of enzymatic activity. Inhibition of protein synthesis in vitro by

Abbreviations used: DSS, disuccinimidyl suberate; DMF, dimethylformamide; BME, beta mercaptoethanol; DTT, dithiothreitol.

0006-291X/82/210007-07$1.00/0

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intact ricin starts only after a lag. In contrast, no lag period is observed with purified A chain. These differences in the time course of protein synthesis inhibition in vitro could be explained by the assumption that only free A chain is able to inactivate ribosomes.

We wished to determine whether reduction of the disulfide bridge is sufficient for inhibition of protein synthesis, or if the two chains also need to be physically separated. We prepared a cross-linked ricin using disuccinimidyl suberate (DSS), a homobifunctional reagent which reacts with primary amino groups, and examined the ability of this cross-linked derivative to inhibit protein synthesis in vitro.

MATERIALS AND METHODS

Materials: Ricin and its A chain were prepared as described (8). DSS was from Pierce. A stock solution 5×10^{-3}M in dimethylformamide was prepared. Flourescamine was from Roche, polyuridylic acid, yeast t-RNA, ATP, GTP, creatine phosphate, creatine phosphokinase were from Sigma, L-[^{3}H]-phenylalanine (20 Ci/mmol) was from Amersham.

Preparation of CL-ricin: Ricin (5×10^{-5}M) was reacted with a 10-fold excess of DSS in 0.5M phosphate buffer, pH 8.0, containing 0.1M NaCl and 5 mM lactose, for two hours at room temperature.

Purification of CL-ricin: All procedures were performed at 4°C, unless otherwise indicated. Excess unreacted reagent was removed by chromatography on a Sephadex G-25 column, using 0.5M sodium phosphate, pH 6.8, containing 200 mM NaCl (PBS) and 0.02% azide. The pooled protein fractions were dialyzed against PBS plus azide to remove remaining lactose, then applied to a Sepharose 4B column (1.5x40cm). The column was then washed extensively with PBS plus azide. A chain not linked to B chain by a non-reducible bond was then eluted with 0.1M Tris-HCl, pH 8.0, containing 0.5M β-mercaptoethanol (BME) and 0.1 mM EDTA. After again washing the Sepharose 4B column with PBS plus azide, CL-ricin and B chain were eluted with 0.1M lactose in PBS. The protein fractions were pooled, concentrated to 1-2 mg/ml, reduced with 100 mM dithiothreitol (DTT) for three hours at 37°C, and applied to a Sephadex G-75 column. Elution was performed with PBS without azide.

Ricin A modification: Ricin A-chain (2×10^{-5}M) was reacted with a 25-fold excess of DSS in 0.5M phosphate buffer, pH 8.0, 0.1M NaCl, 5 mM lactose for two hours at room temperature. The reaction mixture was dialyzed extensively against PBS without azide. No polymerization occurred. The number of primary amino groups remaining after modification of ricin A chain was determined by the flourescamine method (9), using native ricin A as a standard.

Electrophoresis: Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Laemmli (10). Samples were incubated for 15 min at 37°C in 1% SDS, 10% glycerol, 0.2 mM EDTA, 50 mM Tris-HCl, pH 6.8 (SDS sample buffer), in the presence or absence of 1% BME before electrophoresis. Gels were fixed, stained, and destained as described by Fairbanks et al. (11).

Cell-free protein synthesis: Rat liver ribosomes were isolated and treated in separate steps with 0.5% sodium deoxycolate and 0.5M KCl-0.2 mM

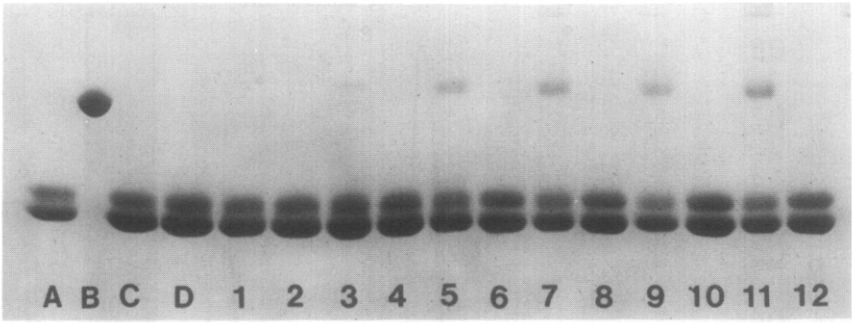

Figure 1. SDS-polyacrylamide gel electrophoresis of ricin treated with the cross-linking agent DSS. Ricin was incubated for various times with either equimolar or 10-fold molar equivalents of DSS in DMF at room temperature. Controls were incubated for 24 hours with an appropriate amount of DMF. The reaction mixtures, unless noted, were reduced for 15 min in SDS sample buffer containing 1% BME prior to electrophoresis. Control samples are: ricin (lane A), unreduced ricin (lane B); ricin treated with 10% DMF (lane C), ricin treated with 1% DMF (lane D). The reaction mixtures are in lanes 1 through 12: 10-fold DSS (odd numbered lanes), or equimolar DSS (even numbered lanes). The incubation times are: 0 min (lane 1 and 2), 5 min (3 and 4), 30 min (5 and 6), 1 hr (7 and 8), 2 hr (9 and 10), 24 hour (11 and 12).

puromycin as described by Cawley et al. (12). The concentration of ribosomes was determined assuming 15.33 pmole/A_{260} unit (13,14). Rat liver S-100 was prepared using the method of Staehelin and Falvey (15). Polyuridylic acid-directed protein synthesis was assayed as described (12).

RESULTS

Preparation of cross-linked ricin: Reaction conditions were chosen to give the highest yield of cross-linked material, while minimizing the formation of polymers and the degree of amino group modification. The gel shown in figure 1 illustrates the results of one experiment performed to determine the optimal reaction conditions. Ricin ($5x10^{-5}$M) was reacted with a 10-fold molar excess of DSS (odd numbered lanes) or an equimolar amount of the reagent (even numbered lanes). At the indicated times lysine (final concentration of $5x10^{-3}$M) was added to stop the reaction. Efficient cross-linking occurred only with a 10-fold excess of reagent. The CL-ricin used in the subsequent experiments was prepared using $5x10^{-5}$M ricin, a 10-fold excess of DSS, and a reaction time of two hours at room temperature. Lactose (5 mM) was included during the cross-linking reaction to protect the B chain binding site. Higher concentrations of lactose inhibited the cross-linking reaction.

Purification of CL-ricin: The purification procedure was designed to select for cross-linked ricin molecules which are still able to bind to

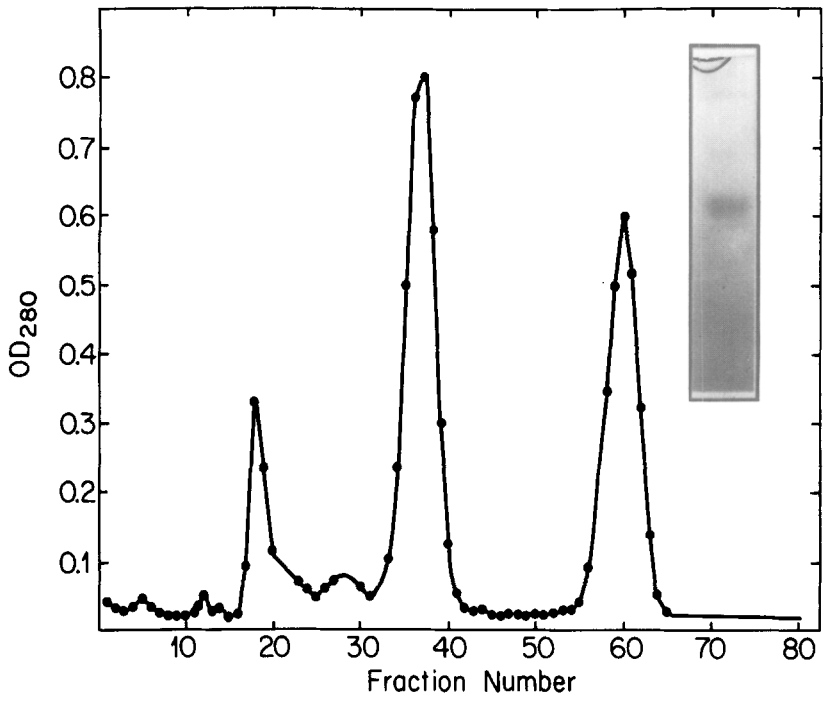

Figure 2. Chromatography of CL-ricin and B chain mixture on Sephadex G-75. The protein fractions obtained from the elution of the Sepharose 4B column with PBS containing 0.1M lactose were pooled, concentrated to about 2 mg/ml, and reduced with 100 mM DTT for 3 hr at 37°C. One ml was applied to a Sephadex G-75 column (1.5x40 cm), previously equilibrated with PBS without azide. Elution was performed at 4°C with the same buffer, and 1.5 ml fractions were collected. Inset: SDS-polyacrylamide gel electrophoresis of purified CL-ricin. The sample was incubated for 15 min at 37°C in SDS sample buffer in the presence of 1% BME prior to electrophoresis.

galactose residues and, thus, have very likely maintained their native B chain conformation. Approximately 95% of the protein bound initially to the Sepharose 4B column. The final elution with lactose gave a mixture of CL-ricin and B chain that could be easily separated on a Sephadex G-75 column (Fig. 2). CL-ricin eluted with the void volume, while the B chain was retarded. The third peak contained DTT. After reduction with 1% BME the purified CL-ricin ran as a single band in SDS-polyacrylamide gel electrophoresis (Fig. 2, insert), co-migrating with unreduced ricin (data not shown). We obtained purified CL-ricin in 10% yield with respect to the starting material.

Cell-free protein synthesis: We compared the ability of reduced CL-ricin, ricin, ricin A, and DSS-ricin A to inhibit poly(U)-directed protein synthesis in a rat liver system (Fig. 3). Reduced CL-ricin, at concentrations as high as 500 ng/ml, did not show any inhibitory activity. Only at a

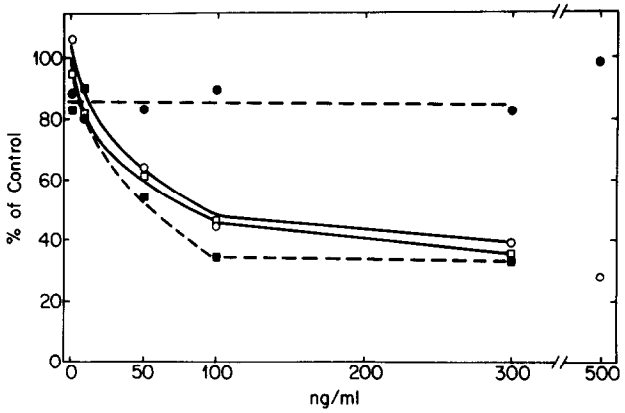

Figure 3: Inhibition of polyuridylic acid translation in a rat liver cell-free system. The toxins were reduced by treatment with 1% BME in 2.5 mM phosphate buffer, pH 7.5, for 30 min at 37°C. Ribosomes (2.5 pmoles) were preincubated with various concentrations of toxins for 10 min at 37°C. The rest of the cell-free system components were then added, and the reaction mixture was incubated for 45 min at 37°C. Ricin (○), CL-ricin (●), ricin A (□), DSS-A (■).

concentration of 1 µg/ml could some inhibition (25-30%) be observed (not shown). Reduced ricin caused 50% inhibition (ED_{50}) at 90 ng/ml, while the ED_{50} for ricin A was about 80 ng/ml. Modification with DSS did not inactivate the enzyme activity of ricin A. Treatment with the cross-linking agent yielded a ricin A molecule which had 80% of its amino groups modified with DSS, yet was as effective an inhibitor as unmodified A chain. In this experiment the DSS:ricin A chain ratio used for modification was 2.5X greater than the DSS:ricin ratio used to prepare CL-ricin (see Methods).

DISCUSSION

When native ricin is reduced its two constituent polypeptides still interact strongly (16,17). When the purified chains are mixed together, even after carboxymethylation to prevent reoxidation of the sulfhydryl groups, they spontaneously and rapidly reassociate to form a stable complex which (1) co-chromatographs with intact ricin, (2) has a sedimentation velocity similar to that of the native toxin, and (3) is as active as ricin in inhibiting protein synthesis *in vitro*. On the other hand, the toxicity of ricin to mice, and the ability of the toxin to inhibit protein synthesis in intact cells decreases considerably after reduction. The presence of an intact disulfide bond is therefore essential for the *in vivo* toxic activity. However, it is not clear

whether the disulfide bridge remains intact during endocytosis and cytoplasmic transport of ricin. If it does remain intact, for toxicity to be expressed reduction must then occur in the cytoplasm (6,7). Olsnes and Saltvedt (18) have proposed that, upon reduction of ricin, the A chain is liberated and a conformational change takes place, exposing the A chain active site. However, there is no direct evidence that the two chains must come apart. When ricin is incubated with a rabbit reticulocyte lysate and then analyzed on an SDS-polyacrylamide gel two bands, corresponding to the A and B chains, appear (6,7). Oda and Funatsu (6) observed that the CL-ricin they prepared remains intact after incubation with the lysate. Since their CL-ricin had the disulfide bridge substituted by a covalent bond, and had very low activity, their results suggest that ricin is activated by the reduction of the disulfide bond. However, this evidence does not rule out the possibility that the two chains, after reduction, are still held together by other interactions. We wanted to ask whether toxicity can be elicited by reduction alone, without separation of the A and B chains.

We cross-linked the A and B chains via a covalent, non-reducible bond with DSS, a reagent which reacts with free amino groups. The disulfide bridge between the two subunits was left intact during the cross-linking. Although the complete amino acid sequence of ricin has been determined (19,20), the spatial relationships of the 9 lysines (7 in the B chain and 2 in the A chain) and the 2 amino terminal groups has not been determined. We have not determined which amino groups reacted. Although it is improbable that the ricin subunits can be cross-linked through many different amino groups, it is possible that there may be some heterogeneity, due to different cross-linking sites, in our CL-ricin preparation.

The CL-ricin, when added to the ribosomes after reduction, had very little inhibitory activity for translation of polyuridylic acid (Fig. 3). The residual activity we observed with 1 μg/ml of CL-ricin (data not shown) could be explained by the presence of a trace of contaminating native ricin. It is

also possible that proteolytic activity present in the cell-free system might cleave the molecule so that an active fragment of the A chain is liberated.

The non-reducible bridge introduced in the ricin molecule contains six methylene groups, and has an approximate length of 11 Å. This should allow considerable motion of the two chains with respect to one another. Since we do not know the position of the cross-linking bond, we cannot exclude the possibility that the B chain is linked to the A chain such that it can sterically hinder the expression of the A chain's enzymatic activity. In any case our results suggest that, in addition to cleavage of the disulfide bridge, other interactions between the two ricin subunits have to be disrupted in order to elicit the activity of the A chain.

ACKNOWLEDGEMENTS

This work was supported by NIH award NIGMS 24797 and DOE contract DE AM03 76 SF00012.

REFERENCES

1. Sandvig, K., Olsnes, S., and Pihl, A. (1976) J. Biol. Chem. 251, 3977-3984.
2. Olsnes, S., Fernandez-Puentes, C., Carnesco, L., and Vazquez, D. (1975) Eur. J. Biochem. 60, 281-288.
3. Hedblom, M.L., Cawley, D.B., Boguslawski, S., and Houston, L.L. (1978) J. Supramol. Struct. 9, 253-269.
4. Sandvig, K., Olsnes, S., and Pihl, A. (1978) Eur. J. Biochem. 82, 13-23.
5. Olsnes, S., Refsnes, K., and Pihl, A. (1974) Nature (London) 249, 627-631.
6. Oda, T., and Funatsu, G. (1979) Agric. Biol. Chem. 43, 547-554.
7. Olsnes, S., Sandvig, K., Refsnes, K., and Pihl, A. (1976) J. Biol. Chem. 251, 3985-3992.
8. Cawley, D.B., Hedblom, M.L., and Houston, L.L. (1976) Arch. Biochem. Biophys. 190, 744-755.
9. Udenfriend, S., Stein, S., Boehlen, P., Darman, W., Leingruber, W., and Weigele, M. (1972) Science 178, 871-872.
10. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
11. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
12. Cawley, D.B., Hedblom, M.L., Hoffman, E.J., and Houston, L.L. (1977) Arch. Biochem. Biophys. 182, 690-695.
13. Baliga, B.S., and Munro, H.N. (1972) Biochim. Biophys. Acta 277, 368-383.
14. Hamilton, M.G., Pavlovec, A., and Petermann, M.L. (1971) Biochemistry 10, 3424-3427.
15. Staehelin, T., and Falavey, A.K. (1971) Methods in Enzymol. 20, 433-449.
16. Lappi, D.A., Kapmeyer, W., Beglau, J.M., and Kaplan, N.O. (1970) Proc. Natl. Acad. Sci. 75, 1096-1100.
17. Houston, L.L. (1980) Bioch. Biophys. Res. Comm. 92, 319-326.
18. Olsnes, S., and Saltvedt, E. (1975) J. Immunol. 14, 1743-1748
19. Funatsu, G., Yoshitake, S., and Funatsu, M. (1978) Agric. Biol. Chem. 42, 501-503.
20. Funatsu, G., Kimura, M., and Funatsu, M. (1979) Agric. Biol. Chem. 443, 2221-2224.