Protection and Rescue of Ribosomes from the Action of Ricin A Chain†

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ABSTRACT: The effect of ricin A chain on the translation of synthetic and natural mRNA by mammalian and wheat germ ribosomes was studied. Both rat liver and wheat germ ribosomes were inactivated in the absence of soluble factors, nucleotides, and energy-regenerating components; however, rat liver ribosomes were much more sensitive to ricin A chain than wheat germ ribosomes. A high concentration (16 mM) of Mg^{2+} reduced considerably the amount of inhibition of both rat liver and wheat germ ribosomes compared with that observed with 10 mM Mg^{2+}. These results indicate that Mg^{2+} can partially restore activity of A chain treated ribosomes, perhaps as a result of an induced conformational change. At high Mg^{2+} concentrations, GTP was able to partially overcome A chain inhibition of protein synthesis. Although the combination of EF-2 and GTP did not prevent the binding of [^{3}H]NEM-labeled A chain to rat liver ribosomes, EF-2 and guanyl-5′-yl imidodiphosphate, a nonhydrolyzable GTP analogue which stabilizes the binding of EF-2 to ribosomes, did prevent binding of A chain. Increasing concentrations of yeast tRNA, in the absence of nucleotides and elongation factors, inhibited A chain binding to rat liver ribosomes and protected ribosomes from inactivation. Poly(uridylic acid) had no effect on binding. The protective effects of EF-2 and tRNA suggest that the binding site of the A chain and the site of the enzymatic lesion are the same and that ribosomes that contain EF-2 or tRNA are inaccessible to A chain and cannot be inactivated except during specific phases of protein synthesis.

Ricin and abrin are toxins isolated from the seeds of *Ricinus communis* and *Abrus precatorius*, respectively, and they appear to have an identical mechanism of action (Olsnes & Pihl, 1976). The ricin and abrin A chains catalytically inactivate eucaryotic 80S ribosomes by damaging a site on the 60S subunit (Sperti et al., 1973). Both apparently inhibit the elongation factor dependent GTPase activity of the ribosome (Benson et al., 1975). The rate of loss of poly(U)[1] translational activity seems to correlate with the loss of GTPase activity, although the extent to which the GTPase can be inhibited is significantly less than the extent of reduction of poly(U) translation (Benson, 1977). The activity of damaged ribosomes can be partially restored under certain conditions. High concentrations of magnesium have been shown to partially restore the poly(U) translational ability of abrin A chain treated ribosomes, and there is preliminary evidence that high concentrations of magnesium can restore the GTPase activity of damaged ribosomes (Skorve et al., 1977).

It is possible to directly block the inactivation of ribosomes by ricin A chain. High concentrations of EF-2 prevent inactivation of ribosomes by ricin A chain (Fernández-Puentes et al., 1976), and it is interesting that ricin A chain also inhibits the binding of EF-2 to the ribosome (Nolan et al., 1976). Since we have shown that ricin A chain specifically binds to a single site on the 60S subunit of rat liver ribosomes (Hedblom et al., 1976, 1979), this suggests that the ricin A chain binding site might overlap the EF-2 binding site. Here we demonstrate that EF-2 as well as tRNA inhibit the binding of A chain and examine the conditions which maximally restore the activity of plant and animal ribosomes.

Materials and Methods

Purification of Ricin A Chain. $Ricin_1$, one of the three species of ricin that can be separated by CM Bio-Gel chromatography, was used as the source of A chain and was purified as previously described (Cawley et al., 1978). $Ricin_1$ was reduced with 2-mercaptoethanol, and the A and B chains were separated by using DEAE Bio-Gel A chromatography with 10 mM galactose included in the column buffer. The A chain preparation contained a single protein as evidenced by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Preparation of Rat Liver and Wheat Germ Ribosomes and S-100. Crude rat liver polysomes were obtained by using the magnesium precipitation method of Palmiter (1974), except that only 4 mL of homogenization buffer was included per g of tissue. The crude ribosomal pellets were resuspended in Staehelin & Falvey (1971) buffer A at a concentration of 100–200 A_{260} units/mL and clarified by low-speed centrifugation (30 min at 10000 rpm) in a Sorvall SS-34 rotor. For general use in protein synthesis, the clarified rat liver ribosomes were treated with 1% sodium deoxycholate for 30 min at 4 °C. The ribosomes were collected by overnight centrifugation at 39 000 rpm in a Beckman type 40 rotor through an equal volume of a 1 M sucrose pad containing buffer A. The resulting pellets were resuspended in buffer A to a concentration of 100–200 A_{260} units/mL, clarified by low-speed centrifugation, and stored in small aliquots at −80 °C.

Salt-washed rat liver ribosomes were obtained from ribosomes prepared by the Palmiter procedure or from deoxycholate-treated ribosomes as described previously (Cawley et al., 1978). To obtain monosomes, the ribosomes prepared by the Palmiter method were treated in separate steps with 25 ng/mL pancreatic RNase A (Sigma) for 10 min at 37 °C and with 0.2 mM puromycin–0.5 M KCl (Blobel & Sabatini,

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[1] Abbreviations used: poly(U), poly(uridylic acid); poly(Phe), poly(phenylalanine); EF-2, elongation factor 2; EF-1, elongation factor 1; buffer A, 20 mM Tris-HCl, pH 7.6, 100 mM NH_4Cl, 5 mM magnesium acetate, 1 mM dithiothreitol; PBS, 5 mM sodium phosphate containing 200 mM NaCl; TMV, tobacco mosaic virus; GDP(NH)P, guanyl-5′-yl imidodiphosphate; NEM, *N*-ethylmaleimide; $NaDodSO_4$, sodium dodecyl sulfate.

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1971). The 80S ribosomes were then collected by ultracentrifugation, clarified, and frozen.

Rat liver S-100 was prepared by using the method of Staehelin & Falvey (1971) and dialyzed into buffer A. The dialyzed preparation was clarified by low-speed centrifugation and stored at −80 °C in small volumes.

Wheat germ ribosomes were isolated from freshly milled wheat germ (ADM Mills, Kansas City, MO) according to the method of Marcus (1972) and, if appropriate, were salt-washed by using the same procedure as described for rat liver ribosomes.

Wheat germ S-100 was prepared as described by Marcus (1972) and centrifuged three times at 65 000 rpm for 5 h to ensure complete removal of the ribosomes. The supernatant from the upper two-thirds of the centrifuge tube was dialyzed twice against 100 volumes of buffer A, clarified by low-speed centrifugation, and stored at −80 °C.

Rat liver EF-2 was a generous gift from Dr. Elizabeth Maxwell of the National Institutes of Health, Bethesda, MD.

Preparation of Wheat Germ and Mouse L Cell Lysates for Translation of mRNAs. Wheat germ S-23 was prepared essentially according to Roberts & Paterson (1973), except that the preincubation was omitted. Mouse L cells were grown in milk dilution bottles as previously described (Barnes et al., 1974) and were the kind gift of Dr. Paul Kitos and Greg Landes. An L cell S-10 extract prepared according to the method of Skup & Millward (1977) was used without micrococcal nuclease treatment.

Preparation of Poly(U)-Sepharose. The method used was one originally developed by Wagner et al. (1971) for the preparation of Sepharose-immobilized, double-stranded, synthetic, nucleic acid polymers. Packed, autoclaved Sepharose Cl-6B (15 mL) was activated for 5 min at 2 °C in 45 mL (total volume) of 0.67 M Na_2CO_3 containing 6 g of cyanogen bromide. The activated gel was washed copiously with 0.1 M $NaHCO_3$ and then with water. The activated Sepharose was gently shaken on a wrist action shaker for 48 h at 4 °C with 55 mg of type II poly(uridylic acid) (Sigma) dissolved in 30 mL of 0.2 M 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0). The derivatized gel was collected on a Buchner funnel and washed extensively with H_2O, 0.5 M NaCl containing 1% $NaDodSO_4$, and finally with 0.5 M NaCl. This poly(U)-Sepharose retained 2 A_{260} units of wheat germ mRNA/mL of packed gel and was far superior in this respect to commercial oligo(dT)-cellulose.

Preparation of Crude Wheat Germ RNA. Reagent grade phenol was redistilled and stored in crystalline form in brown bottles prior to washing with ethylenediaminetetraacetic acid (EDTA) as described by Marcus et al. (1974). Wheat germ (40 g) was ground with an equal weight of sand and 2 volumes of 20 mM Hepes (pH 7.6), 5 mM magnesium acetate, 5 mM EDTA, 20 mM NaCl. The paste was scraped into centrifuge tubes and centrifuged for 10 min at 12 000 rpm. The supernatant was filtered through cheesecloth, 0.1 volumes of 1 M sodium acetate (pH 5.2) and 10% $NaDodSO_4$ were added, and RNA was extracted using phenol–chloroform (Palmiter, 1974). Crude RNA was precipitated by adding 2 volumes of absolute ethanol to the combined aqueous layers from two extractions and allowing the solution to remain at −5 °C overnight. Forty grams of wheat germ typically yielded about 6500 A_{260} units of crude RNA.

Isolation of mRNA from Wheat Germ, Rat Liver, and Tobacco Mosaic Virus. All glassware that came in contact with RNA or cell-free protein synthesis lysates was acid-washed, rinsed with twice-distilled water, heated briefly to 250 °C, and autoclaved. All solutions were made with water that had been boiled briefly in the presence of diethyl pyrocarbonate to destroy RNase.

Poly(U)-Sepharose chromatography was performed essentially according to Krystosek et al. (1975). Up to 6500 A_{260} units of crude wheat germ RNA or about 500 A_{260} units of crude rat liver polysomes was dissolved in 0.5 M NaCl–0.5% $NaDodSO_4$ and applied to a 1.8 × 7 cm column of poly(U)-Sepharose equilibrated at room temperature in the same buffer. The column was washed with 0.5 M NaCl–0.5% $NaDodSO_4$ until the A_{260} of the effluent was about 0.1; the column was then washed with 0.5 M NaCl until no $NaDodSO_4$ could be precipitated from the effluent by the addition of 0.2 M KCl. The column was then eluted with sterile distilled water. The poly(A)-containing (mRNA) fractions were pooled, made 0.1 M in NaCl, and precipitated overnight with 2.5 volumes of absolute ethanol at −5 °C. The precipitated mRNA was collected by centrifugation (10 min at 10 000 rpm in a Sorvall SS-34 rotor), dried under a gentle stream of nitrogen, resuspended in sterile distilled water to 20–40 A_{260} units/mL, and was stored in small volumes at −80 °C. Typical yields were 20–25 A_{260} units of wheat germ poly(A)-containing RNA (0.3–0.5% of total RNA) and 5–10 A_{260} units of rat liver mRNA (1–2% of total polysomal RNA).

Tobacco mosaic virus (TMV) was the kind gift of Dr. Milton Gordon of the Department of Biochemistry at the University of Washington, Seattle. TMV RNA was prepared using the phenol–EDTA method of Marcus et al. (1974).

Poly(U)-Directed Protein Synthesis. Reaction mixtures of 50 μL were assayed as described previously (Cawley et al., 1977). When ribosomes were preincubated with ricin A chain, the mixture (24 μL) contained: 25 mM Tris–acetate, 50 mM potassium acetate, 1 mM $CaCl_2$, magnesium acetate (as indicated in the figure legends), 42 μM dithiothreitol, 6 mM NaCl, and 0.15 mM sodium phosphate. After a 10-min exposure at 37 °C to the ricin A chain or buffer, anti-ricin antibody (1 μL) was added and the assay tubes were made up to 50 μL with the components necessary for poly(phenylalanine) synthesis as described previously (Hedblom et al., 1979). The assay mixture was incubated for 45 min at 37 °C and the hot Cl_3CCOOH insoluble radioactivity determined. The counting efficiency for tritium was 42%.

Ribosome concentrations were determined by assuming 15.33 pmol/A_{260} unit for rat liver ribosomes (Hamilton et al., 1971; Baliga & Munro, 1972) and 15.62 pmol/A_{260} unit for wheat germ ribosomes (Bonner, 1965; Berridge et al., 1970).

mRNA-Directed Cell-Free Protein Synthesis. A 50-μL reaction mixture for wheat germ cell-free translation contained: 10–15 μL of wheat germ S-23 extract (containing 1.5 A_{260} units), 110 mM potassium ion (24–30 mM as KCl, 80–90 mM as potassium acetate), magnesium acetate (as indicated in the individual experiments), 25 mM Hepes (pH 7.6), 5 mM 2-mercaptoethanol, 10 mM ATP, GTP as indicated, 10 mM creatine phosphate, 30 μM of each of 19 amino acids (excluding leucine), 2 μg of creatine phosphokinase, 2.5 μCi of [^{3}H]leucine (Amersham/Searle, 120 Ci/mmol), and a saturating amount (0.1–0.2 A_{260} unit or about 5 μg) of wheat germ mRNA. TMV RNA was translated as described by Roberts & Paterson (1973).

The L cell S-10 lysate contained a considerable amount of endogenous mRNA. For the assays performed here, no attempt was made to remove this mRNA. The L cell lysate was supplemented with wheat germ mRNA until the system was saturated. Background radioactivity (that obtained in the absence of protein synthesis) was determined in both poly(U)

and mRNA directed protein synthesis experiments by including 1 mM cycloheximide (Boehringer) in the assay. The L cell lysate system contained, in a final volume of 50 μL: 30 μL of S-10 extract (4.3 A_{260} units), 1 mM ATP, 10 mM creatine phosphate, 2 μg of creatine phosphokinase, 0.5 mM GTP, 5 μM each of 19 exogenous amino acids excepting leucine, 80 mM KCl, 30 mM Hepes, pH 7.6, 4 mM magnesium acetate, 1.2 mM dithiothreitol, and 2.5 μCi (about 1 μM) of [^{3}H]leucine.

In these experiments, the ricin A chain was added directly to the reaction mixtures containing wheat germ and L cell lysates. The assays were performed at 27 °C for the times indicated in the figure legends. The reactions were stopped with 0.2 mL of 0.1 N KOH, and the samples were incubated for 15 min at room temperature. An equal volume of 10% trichloroacetic acid was added and the samples were filtered and counted as described above.

Preparation of Antibodies to Ricin. New Zealand white rabbits were immunized with formalin-treated ricin by using the method of Olsnes & Saltvedt (1975). The precipitate from 50% ammonium sulfate fractionation of the antiserum was dissolved in PBS buffer, pH 6.5, and dialyzed exhaustively against the same buffer. After dialysis any insoluble material was removed by low-speed centrifugation, and the fractionated antibody preparation was stored at −80 °C in small volumes at a concentration of 81 mg/mL. Four micrograms of this antibody preparation was capable of neutralizing a minimum of 10 ng of purified ricin A chain, judging from its ability to completely prevent ribosome inactivation by the A chain.

Preparation of a [^{3}H]-N-Ethylmaleimide Derivative of the Ricin A Chain and Ribosome Binding Assay. Three milligrams of ricin A chain was reacted at neutral pH with a 20-fold molar excess of *N*-[*ethyl*-2-^{3}H]maleimide (New England Nuclear, 150 Ci/mmol) for 1.5–2 h at room temperature, resulting in the incorporation of 0.85–1.0 mol of reagent per mol of A chain. This derivatized A chain inhibited rat liver ribosomes in a manner identical with that of untreated A chain (Hedblom et al., 1979).

The binding of [^{3}H]NEM-labeled A chain to rat liver ribosomes was determined as follows. About 20 pmol of rat liver ribosomes was reacted with an approximately sevenfold molar excess of labeled A chain in a final reaction volume of 200 μL containing 25 mM Tris–acetate (pH 7.6), 50 mM potassium acetate, 1.0 mM $CaCl_2$, and NaCl and magnesium acetate as indicated in the table legends. At the end of 10-min incubation at 37 °C, 175-μL samples were centrifuged for 30 min at 165 000*g* in a Beckman Airfuge (30 psig air pressure). The supernatant was carefully removed and the tip of each nitrocellulose tube was cut off with a razor blade and placed in a 10 × 75 mm test tube. The ribosome pellet was solubilized in two successive 50-μL portions of 3% sodium dodecyl sulfate by heating the tube to 90 °C and vigorously shaking on a Vortex mixer. The combined 100-μL sample was counted in 5 mL of Brays solution with a counting efficiency of 41% for ^{3}H.

Results

Effect of Magnesium upon A Chain Inhibition of Protein Synthesis. The data in Figure 1A show that rat liver ribosomes treated with 0.4 ng/mL of ricin A chain were inhibited 85% in poly(U) translation when assayed in 10 mM Mg^{2+}, but only 55% in 16 mM Mg^{2+}. Poly(U) translation by wheat germ ribosomes treated with 20 μg/mL A chain was inhibited 60% when assayed in the presence of 10 mM Mg^{2+}, but only 27% when assayed in 18 mM Mg^{2+} (Figure 1B). Because poly(U) translation assays only elongation processes, the effect of ricin

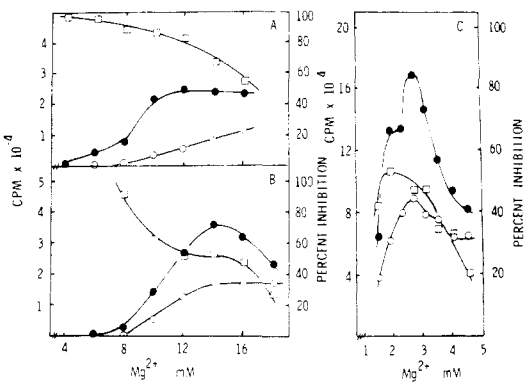

FIGURE 1: Magnesium dependency of ricin A inhibition of translation. (A) Inhibition of poly(U) translation by 0.4 ng/mL ricin A chain (○) was tested with 2.5 pmol of rat liver ribosomes at various Mg^{2+} concentrations as described under Materials and Methods. Identical assays without A chain (●) were used as controls. The assays were terminated after 45 min at 37 °C. The open squares (□) are the percent inhibition of synthesis by the A chain treated ribosomes compared with the control at the same Mg^{2+} concentration. (B) The assays and symbols are the same as in panel A, except that 20 μg/mL ricin A chain was used with 2.5 pmol of wheat germ ribosomes. (C) Inhibition by 5 μg/mL ricin A chain was tested by using a natural message isolated from wheat germ and a wheat germ cell-free system described under Materials and Methods. The background radioactivity obtained in the presence of 1 mM cycloheximide was 2990 cpm. The symbols are as indicated for panels A and B.

A chain on mRNA translation was examined when the magnesium concentration was varied over a more physiological range (1.5–4.5 mM). The results (Figure 1C) with the wheat germ system show that 5 μg/mL of ricin A chain caused about 45% inhibition of protein synthesis from 2 to 3 mM Mg^{2+}, but the inhibition dropped rapidly above 3.5 mM until only 20% inhibition of mRNA translation was observed at 4.5 mM Mg^{2+}.

Effect of GTP upon Protein Synthesis Inhibition by the A Chain. The effect of GTP was additive to the effect afforded by high concentrations of magnesium alone. At 17 mM Mg^{2+} (Figure 2, bottom), A chain treated wheat germ ribosomes assayed in the presence of GTP (△) were more active than A chain treated ribosomes assayed in the absence of GTP (○). Therefore, at this concentration of Mg^{2+}, GTP, which was present only during the translation of poly(U), was able to partially overcome the A chain inhibition of protein synthesis. However, at 10 mM Mg^{2+}, the same level of A chain inhibition was observed in the presence (▲) and absence (●) of GTP. At 13 mM Mg^{2+}, GTP gave an intermediate effect (data not shown). Figure 2 (top) shows that GTP had little or no effect upon the wheat germ mRNA-directed protein synthesis by an unfractionated wheat germ lysate treated with various concentrations of ricin A chain and assayed in the presence of 2.3 mM Mg^{2+}. Fernández-Puentes et al. (1976) found essentially no protection of rabbit reticulocyte ribosomes by 250 μM GTP against ricin A chain.

Translation of wheat germ or rat liver mRNA or tobacco mosaic virus RNA by wheat germ lysate did not require exogenous GTP; however, the dependence on added GTP was increased considerably by washing rat liver or wheat germ ribosomes in high salt. While A chain treated ribosomes from rat liver and wheat germ were less inhibited when assayed in the presence of GTP, the effect was much more pronounced in the wheat germ system (Table I). The data demonstrate that, in the presence of GTP, wheat germ ribosomes are about 20 000 times less sensitive to A chain than rat liver ribosomes.

Effect of GTP, GDP(NH)P, and EF-2 on A Chain Binding to Rat Liver Ribosomes. Because the A chain can block the

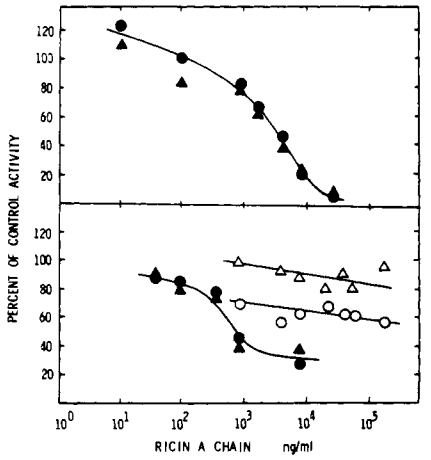

FIGURE 2: Mg^{2+} dependency of the GTP protection from ricin A chain inhibition. (Bottom) Poly(U) translation. Wheat germ ribosomes (2.4 pmol, not salt washed) were incubated in the presence of various amounts of ricin A chain for 10 min at 37 °C, and the A chain was neutralized by addition of anti-ricin serum as described under Materials and Methods. The ribosomes were then assayed for 45 min at 37 °C as described under Materials and Methods. GTP, when included, was 0.2 mM and was not present during the initial ricin treatment. Each assay was run in triplicate. The magnesium concentration was the same in the preincubation and during the subsequent assay. The activity in the absence of poly(U) was 515 cpm (− GTP) and 559 cpm (+ GTP). Control synthesis in the presence of poly(U) was 23 670 cpm (− GTP) and 27 290 cpm (+ GTP). The open symbols represent synthesis in the presence of 17 mM Mg^{2+} without GTP (○) or with GTP (△), and the closed symbols represent synthesis in the presence of 10 mM Mg^{2+} without GTP (●) or with GTP (▲) included in the reaction mixture. (Top) Wheat germ mRNA directed protein synthesis by a wheat germ cell-free system. The assay was performed as described under Materials and Methods for 90 min at 27 °C in the presence of 2.3 mM Mg^{2+} and the indicated amounts of ricin A chain. The concentration of GTP was 0.1 mM when included, and 0.1 A_{260} unit of wheat germ mRNA was added to each 50-μL assay tube. The background radioactivity in the presence of cycloheximide was 3750 cpm in the absence or presence of GTP, while the control activities without A chain were 132 200 cpm (− GTP) and 167 400 cpm (+ GTP). The symbols represent synthesis in the absence of GTP (●) and in the presence of GTP (▲).

Table I: Ricin A Chain Inhibition of Poly(U) Translation by Wheat Germ and Rat Liver Ribosomes in the Presence and Absence of Added GTP[a]

cell-free system	A chain concn (ng/mL)	% inhibn of poly(Phe) synthesis + GTP	% inhibn of poly(Phe) synthesis − GTP
wheat germ	13000	38	90
wheat germ	1300	11	50
rat liver	2.9	53	68
rat liver	0.6	45	62

[a] Salt-washed ribosomes (2.4 pmol) were preincubated 10 min at 37 °C in the presence of the indicated concentrations of ricin A chain, the A chain was neutralized by addition of anti-ricin serum, and the ribosomes were then assayed for poly(U) translation at 37 °C for 45 min. The magnesium concentration was 17 mM during the preincubation and during the subsequent protein synthesis assay, and GTP was present at a concentration of 0.2 mM where indicated. The level of phenylalanine incorporation in control samples without the addition of A chain was used to calculate the percent inhibition of synthesis.

binding of EF-2 to the ribosome (Nolan et al., 1976), the effect of GTP and EF-2 on the A chain binding was examined (Table II). The binding of [^{3}H]NEM-labeled A chain to rat liver monosomes in 7.5 mM Mg^{2+} was unaffected either by GTP or by EF-2 alone. Furthermore, EF-2 and GTP together did not significantly inhibit the binding of the labeled A chain.

Table II: Binding of Ricin A Chain to Rat Liver Ribosomes[a]

expt	additions to binding assay	net cpm bound to ribosome pellet	% of control binding
one	none	842	100
	EF-2	1044	124
	GDP(NH)P (0.5 mM)	940	112
two	none	900	100
	EF-2 + GTP (0.2 mM)	858	95
	EF-2 + 0.2 mM GDP(NH)P	482	54

[a] Rat liver monosomes (20 pmol) were preincubated for 20 min at 37 °C in the presence of (final concentrations) 37.5 mM Tris-acetate (pH 7.6), 50 mM potassium acetate, 1 mM $CaCl_2$, 7.5 mM magnesium acetate, 0.5 mM dithiothreitol, 50 μM EDTA, EF-2 (100 μg), and guanine nucleotide was added as indicated. [^{3}H]-NEM-labeled A chain (139 pmol; 92 cpm/pmol) was subsequently added and the reaction mixture incubated for 10 min at 37 °C. After the addition of labeled A chain (in PBS), the concentration of NaCl was 5 mM. All assays were done in duplicate, and the average values are shown.

Table III: Inhibition of A Chain Binding to Rat Liver Ribosomes by tRNA[a]

tRNA concn (mg/mL)	net cpm A chain bound to ribosomal pellet	% of control binding
0	965	100
0.24	786	81
2.4	488	51
24.0	249	26

[a] [^{3}H]NEM-labeled A chain (140 pmol) was reacted for 10 min at 37 °C with 19.3 pmol of rat liver ribosomes in the presence of 5 mM NaCl, 7.5 mM magnesium acetate, and various amounts of yeast tRNA. The A chain–ribosome complexes were isolated by centrifugation, and the amount of binding was determined as described under Materials and Methods.

The combination of EF-2 and GDP(NH)P, a nonhydrolyzable GTP analogue, caused 50% inhibition of the binding of [^{3}H]NEM-labeled A chain to ribosomes, while GDP(NH)P alone caused no significant inhibition (Table II).

The inclusion of bovine serum albumin or ovalbumin (1 mg/mL final concentration) or 20 μL of rabbit serum did not prevent [^{3}H]NEM-labeled A chain from binding to rat liver ribosomes. Bovine serum albumin and ovalbumin increased binding about 25% and there was no effect on the level of binding by using serum.

Effect of tRNA upon Ribosome Binding and Protein Synthesis Inhibition by A Chain. Because tRNA alone can compete with EF-2 for a ribosome binding site (Grasmuk et al., 1977) and since the data above indicate that EF-2 can inhibit the ribosome binding of ricin A chain under some conditions, the ability of tRNA to inhibit the binding of the A chain to the ribosome was examined (Table III). The data demonstrate that increasing concentrations of yeast tRNA, in the absence of either nucleotides or elongation factors, significantly inhibited the binding of [^{3}H]NEM-labeled A chain to rat liver ribosomes. tRNA also protected rat liver ribosomes from A chain inactivation as shown in Table IV. The optimal concentration of tRNA is about 0.1 mg/mL under the conditions used for poly(U) translation. The percent of ricin A chain inhibition of poly(Phe) synthesis is reduced as the concentration of tRNA is increased. While these effects must be interpreted with caution because of the inhibition of total synthesis of tRNA, the most dramatic effect of tRNA

Table IV: Effect of tRNA upon Ribosome Inactivation by the Ricin A Chain[a]

tRNA concn (mg/mL)	cpm of [^{3}H]poly(Phe) synthesized		% control act.
	control	plus A chain	
0	812	1 231	
0.024	26 237	13 251	51
0.048	38 824	18 702	48
0.120	40 315	29 331	73
0.240	20 341	17 497	86
0.600	21 808	17 633	81
1.2	18 687	17 654	94
2.4	14 753	12 488	84
4.8	7 016	6 752	93

[a] Rat liver ribosomes (2.5 pmol) were preincubated for 2 min at 37 °C in the presence of various concentrations of yeast tRNA in 25 mM Tris-HCl (pH 7.6), 11 mM magnesium acetate, 50 mM potassium acetate, and 1 mM $CaCl_2$. Where indicated, ricin A chain was added to a final concentration of 0.4 ng/mL and the incubation continued for an additional 10 min at 37 °C. The ribosomes were then assayed (without addition of antiserum) for poly(U) translation as described under Materials and Methods. The net cpm [^{3}H]poly(Phe) synthesized are shown and are the average of duplicate samples.

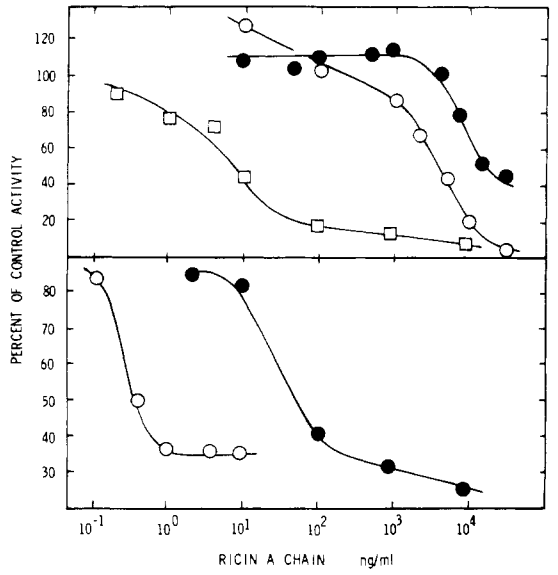

FIGURE 3: Differential sensitivity of plant and mammalian cell-free poly(U) and mRNA translation to ricin A chain. (Bottom) Poly(U) translation. Two and one-half picomoles of salt-washed wheat germ (●) or rat liver (○) ribosomes was preincubated 10 min at 37 °C in the presence of ricin A chain, the A chain was neutralized by using anti-ricin serum, and the ribosomes were assayed for 45 min at 37 °C in the presence of 10 mM Mg^{2+} and 0.2 mM GTP as described under Materials and Methods. Background radioactivity in the absence of poly(U) was 470 cpm for the wheat germ system and 1834 cpm for the rat liver system. These values were subtracted from the activity of the A chain treated samples. The activity of control samples without A chain in the presence of poly(U) was 16 200 cpm (wheat germ) and 43 100 cpm (rat liver). (Top) mRNA translation. Wheat germ and L cell lysates were assayed as described under Materials and Methods for 90 min at 27 °C. The wheat germ system was incubated with 12.2 μg of TMV RNA in the presence of 2.5 mM Mg^{2+} and 0.02 mM GTP (●) or with 0.2 A_{260} unit of wheat germ mRNA in the presence of 4 mM Mg^{2+} and 0.5 mM GTP (○). Cycloheximide background radioactivity for TMV RNA was 2871 cpm and for wheat germ mRNA was 3750 cpm. Control activity for TMV RNA was 65 500 cpm and for wheat germ RNA was 187 300 cpm. The L cell system (□) was incubated with 0.2 A_{260} unit of wheat germ mRNA in the presence of 4.0 mM Mg^{2+} and 0.5 mM GTP. Background radioactivity was 2068 cpm and control activity was 29 650 cpm.

occurred in the range optimal for poly(Phe) synthesis. Although the level of poly(Phe) synthesis was low in the absence of exogenous tRNA, some stimulation was observed in the presence of ricin A chain. When added to binding assays in amounts that saturate the rat liver cell-free protein synthesis under similar conditions, poly(U) (100 μg/mL) had no effect on the binding of the A chain to rat liver ribosomes (data not shown). Ribosomal RNA and tobacco mosaic virus RNA (up to 0.4 mg/mL) did not inhibit the binding of A chain.

Table V: Inactivation of Rat Liver and Wheat Germ Ribosomes by Ricin A Chain[a]

ribosomes	% of inhibn of poly(Phe) synthesis		
	immediately after A chain treatment	after centrifugation and collection of the ribosomes	
		without antiricin	with antiricin
rat liver	40	80	74
wheat germ	35	39	30

[a] Three hundred and fifty-two picomoles of rat liver or wheat germ ribosomes were incubated in 25 mM Tris-HCl (pH 7.6), 10.4 mM magnesium acetate, 50 mM potassium acetate, 1 mM $CaCl_2$, 0.5 mM NaH_2PO_4, and 20 mM NaCl with and without 10.8 μg/mL ricin A chain for 10 min at 37 °C. Samples containing 2.5 picomoles of ribosomes were then withdrawn, anti-ricin serum was added, and the ribosomes were assayed for poly(U) translation as described under Materials and Methods. The remainder of all four ribosome stock solutions was diluted to 5 mL with Staehelin and Falvey buffer A. The ribosome solutions were then layered over a 3-mL 20% sucrose pad in buffer A and centrifuged 4 h at 39 000 rpm in a Beckman type 40 rotor. The ribosomes were resuspended in 7.5 mM Tris-HCl (pH 7.6), 10.4 mM magnesium acetate, 50 mM potassium acetate, and 1 mM $CaCl_2$, clarified by low-speed centrifugation, and 2.5 picomoles was assayed for poly(U) translation in the presence and absence of anti-ricin serum as indicated. Control samples without A chain were used to calculate the percent inhibition.

Differential Sensitivity of Liver and Wheat Germ Cell-Free Protein Synthesis Systems to Inhibition by A Chain. The data shown in Figure 3 (top) demonstrate that, when translation of poly(U) is measured at 10 mM Mg^{2+} and 0.2 mM GTP, wheat germ ribosomes are two orders of magnitude less sensitive to ricin than are rat liver ribosomes. Based upon the concentration of A chain needed to achieve 50% inhibition of protein synthesis, the data indicate that 0.67 fmol of ricin A chain will inactivate 1250 fmol of rat liver ribosomes in 10 min at 37 °C in the presence of 10 mM Mg^{2+} and 0.2 mM GTP. Under the same conditions, 67 fmol of the A chain is required to inactivate the same number of wheat germ ribosomes.

The data (Figure 3, bottom) show that this difference in sensitivity extends to mRNA-directed translation. While a direct comparison of the rates of inactivation cannot be made here because of the crude nature of these lysates, it is obvious that L cell ribosomes are two to three orders of magnitude more sensitive to A chain than are wheat germ ribosomes.

Are Wheat Germ Ribosomes Irreversibly Inhibited by Ricin A Chain? Wheat germ and rat liver ribosomes were treated with ricin A chain and small, representative samples were tested for their ability to synthesize poly(Phe); the remaining ribosomes were pelleted through sucrose to remove the A chain (Hedblom et al., 1976). The sucrose-washed, A chain treated ribosomes were then assayed again for their poly(U) translational ability, both in the presence and absence of anti-ricin antibody. The results (Table V) show that physically removing the A chain did not restore the activity of the wheat germ or rat liver ribosomes; thus, both appear to be irreversibly inactivated by the A chain. It is not clear why the inhibition of the rat liver ribosomes by the A chain differed so much

between the initial assay and the one performed with the centrifuged ribosomes (40% inhibition initially vs. 80% inhibition in the second assay).

On several occasions we have found that ricin A chain stimulates protein synthesis in the wheat germ system; this effect was observed when the A chain was present at concentrations (1–10 ng/mL) well below those required to cause significant inhibition as shown in Figures 2 and 3. We have also observed this effect using rat liver ribosomes at very low concentrations (1–50 pg/mL) of A chain (data not shown). We have observed stimulation of up to 170% in these systems, but this was not observed in all experiments. Others have occasionally observed a definite stimulation of protein synthesis of HeLa cells by using low concentrations of ricin (S. Olsnes, personal communication). If the conditions under which this stimulation was observed could be defined more precisely, it might be possible to use this effect as a tool to probe ricin's mechanism of action.

Discussion

The interaction of ricin A chain with the ribosome can be written as a normal Michaelis–Menten reaction

$$A + R_s \rightleftharpoons R_s{\cdot}A \quad (1)$$

$$R_s{\cdot}A \rightarrow R_p{\cdot}A \quad (2)$$

$$R_p{\cdot}A \rightleftharpoons R_p + A \quad (3)$$

where R_s is the native ribosome (substrate) for ricin A chain (A) and R_p is an inhibited ribosome (product). There is no reason to believe that the lesion introduced at step 2 by ricin A chain is reversible. However, a fourth reaction

$$R_p \rightleftharpoons R_p^* \quad (4)$$

may occur which generates a ribosome whose activity is partially restored. Because the treated ribosomes are inactive, the A chain–ribosome binding that we have observed (Hedblom et al., 1976) probably represents the association of the A chain with a product ribosome (step 3).

Protection of Ribosomes by Ligands That Block A Chain Binding. Our observation that EF-2 prevents binding of A chain is consistent with the facts that ricin A chain blocks binding of EF-2 to mammalian ribosomes (Fernández-Puentes et al., 1976; Montanaro et al., 1975; Carrasco et al., 1975) and that EF-2 can prevent inactivation of ribosomes by A chain (Fernández-Puentes et al., 1976). The better inhibition of A chain binding by EF-2 in the presence of GDP(NH)P compared with GTP is expected since nonhydrolyzable GTP analogues promote stable binding of EF-2 to ribosomes (Nolan et al., 1976). The protective effects of EF-2 and tRNA suggest that they can directly block the initial association of the A chain with the ribosome (step 1) in addition to being able to block the binding that we observe (step 3). It is likely that the A chain binding site is near a ribosomal site with which EF-2 and tRNA interact and therefore that ribosomes which contain EF-2 or tRNA are partially or totally inaccessible to A chain inactivation.

Restoration of Activity of Damaged Ribosomes. Our results suggest that Mg^{2+} can partially restore the poly(U) translation activity of A chain-treated ribosomes, perhaps by inducing a conformational change which leads to a new ribosome species (R_p^*). However, when poly(U) translation is measured in low Mg^{2+}, ribosomes treated with A chain at high Mg^{2+} are inhibited to the same extent as ribosomes treated in low Mg^{2+} (Hedblom et al., 1979). We have found that there is a limit to the amount of inhibition that can be observed in vitro, suggesting that modified ribosomes (R_p) still have a low level of activity. The overall role of Mg^{2+} in maintaining the functional and physical integrity of the ribosome is consistent with the notion that elevated Mg^{2+} concentrations may circumvent A chain induced alterations in the ribosome structure and somehow help to overcome the reduced affinity of the ribosome for elongation factors. This agrees with the fact that high concentrations of elongation factors restore the translational ability of damaged ribosomes (Olsnes et al., 1975).

The apparent stimulation by GTP (in the presence of high Mg^{2+}) of translation by A chain treated ribosomes from wheat germ and rat liver may have resulted from a promotion of the interaction of the binary (EF-2·GTP) and ternary (EF-1·aa-tRNA·GTP) complexes with the damaged ribosomal site(s). The fact that the ribosome-dependent GTPase activity associated with both elongation factors was inhibited by A chain (Benson, et al., 1975; Benson, 1977), even though the EF-1 and EF-2 binding sites may not substantially overlap (Grasmuk et al., 1977), suggests that the GTP moiety of the two complexes is inaccessible to the GTPase of the A chain treated ribosomes. While the A chain may covalently modify the 60S subunit in some as yet unknown way, it is also possible that the A chain may not be a lytic enzyme and may cause only conformational changes in the ribosome that limit or modify the accessibility of the substrate, in this case, the GTP bound to the soluble factor.

Our data are consistent with the concept that the damaged GTPase has restricted accessibility to the elongation factor–GTP complex. The restoration of activity by magnesium, GTP, and elongation factors is probably due to an improvement of the interaction of the factor–GTP complexes with the GTPase site. This implies that ricin does not act upon one particular phase or catalytic reaction of the translational cycle, but rather that it has a more general effect upon translation by causing effects that ultimately deprive the ribosome of GTP. Thus, ricin's action probably affects initiation, elongation, and termination.

Insensitivity of Wheat Germ Ribosomes to the A Chain. While GTP does restore somewhat the activity of inhibited rat liver ribosomes, the activity of damaged wheat germ ribosomes can be restored nearly to control values by GTP (in the presence of high magnesium). Our previous inability (Cawley et al., 1977) to observe inhibition in the wheat germ system by using very high ricin A chain concentrations was due undoubtedly to the powerful restorative effect of GTP at the concentration of magnesium (15.8 mM) used in those experiments. However, even under optimal conditions for inhibition, wheat germ ribosomes are at least 100-fold less sensitive to the A chain than rat liver ribosomes. The fact that wheat germ ribosomes are differentially protected (compared with rat liver) by GTP suggests that the interaction of GTP or the factor–GTP complexes with wheat germ ribosomes may be different from the analogous interactions in the rat liver system. The insensitivity of the wheat germ system is evident also in natural mRNA translation, wherein added GTP has no restorative effect.

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Protein–Water Interactions. Heat Capacity of the Lysozyme–Water System†

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ABSTRACT: Calorimetric measurements of the heat capacity of the lysozyme–water system have been carried out over the full range of system composition at 25 °C. The partial specific heat capacity of the protein in dilute solution is 1.483 ± 0.009 J K^{-1} g^{-1}. The heat capacity of the dry protein is 1.26 ± 0.01 J K^{-1} g^{-1}. The system heat capacity responds linearly to change in composition from dilute solution to 0.38 g of water per g of protein (h) and is an irregular function at lower water content. The break in the heat capacity function at 0.38 h defines the amount of water needed to develop the equilibrium solution properties of lysozyme as being 300 molecules of water/protein molecule, just sufficient for monolayer coverage. The heat capacity behavior at low water content describes three hydration regions. The most tightly bound water (0–0.07 h), probably principally bound to charged groups, is characterized by a partial specific heat capacity of 2.3 J K^{-1} g^{-1}, a value close to that for ice. A heat of reaction associated with proton redistribution is reflected in the heat capacity function for the low-hydration region. Between 0.07 and 0.25 h the heat capacity increases strongly, which is understood to reflect the growth of patches of water covering polar and adjacent nonpolar portions of the protein surface. The hydration shell is completed by condensation of solvent over the weak-interacting portions of the surface, in a process displaying a transition heat.

The interactions between water and protein are generally understood to be important determinants of folding and function. Despite a large literature on protein hydration and related problems (Kuntz & Kauzmann, 1974), some important questions remain unresolved. Heat capacity measurements seem particularly well suited to probing three of these.

First, how much water is needed to fully hydrate a protein? We define the water of hydration, or the bound water, as water perturbed in its properties or that perturbs protein properties through interactions with the protein. Techniques for studying protein hydration each may see only a portion of the total bound water (Richards, 1977). Heat capacity data are likely to reflect all water important for equilibrium considerations,

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