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COMPARISON OF THE PEPTIDE "FINGER PRINT" AND ANTIGENIC SPECIFICITY OF A DIPHTHERIA TOXIN FRAGMENT PRODUCED BY A PHAGE-MUTANT LYSOGEN AND "FRAGMENTS A AND B" OF THE TOXIN

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SUMMARY A toxin fragment produced by a phage-mutant lysogen (C7 (β–NG2)) of *Corynebacterium diphtheriae* and "fragments A and B" of diphtheria toxin protein were purified by polyacrylamide gel electrophoresis. The "finger prints" and antigenic specificities of these purified proteins were studied by peptide mapping and immunodiffusion. Tryptic peptide maps showed that β–NG2 protein had a very similar "finger print" to "fragment A" except that the latter had a few minor additional peptides. These maps were entirely different from that of "fragment B". Immunodiffusion analyses using a horse antitoxin demonstrated that β–NG2 protein appeared to be identical with "fragment A" in terms of antigenic specificity. β–NG2 protein did not cross-react with "fragment B" on immunodiffusion.

INTRODUCTION

Previously, we reported the isolation of a unique mutant (β–NG2) of converting phage β (Matsuda et al., 1972). The lysogen (C7 (β–NG2)) of this mutant produced extracellularly a single major protein species (β–NG2 protein) with a molecular weight of 24,000. The protein had no diphtheria toxicity but had ADP ribosylation activity and cross-reactivity with diphtheria antitoxin. These properties suggested that β–NG2 protein might be a fragment of the diphtheria toxin molecule with very similar properties to those of "fragment A" of the toxin (Gill and Dinius, 1971; Gill and Pappenheimer, 1971; Collier and Kandel, 1971; Drazin et al., 1971). Accordingly the relationship between these proteins related to toxin were studied. This paper describes the results of comparison of β–NG2 protein with "fragments A and B" on the peptide "finger print" and antigenic specificity.

MATERIALS AND METHODS

1. *Preparation and concentration of the β-NG2 protein fraction*

β-NG2 protein was obtained from a phage-mutant lysogen, C7(β-NG2) of *Corynebacterium*

diphtheriae (Matsuda et al., 1972), using a similar system to that described by Hirai et al. (1966) for toxin production by the PW8 strain. Cells of C7(β-NG2) were grown at 36 C in PGT medium (Barksdale and Pappenheimer, 1954) containing 4% maltose and 0.04 μg Fe^{++}/ml. When the OD reached 1.8–2.0, the culture was chilled and centrifuged at 8,000×*g* for 10 min and the packed cells thus obtained were resuspended in CST-6 medium (Uchida and Yoneda, 1967) at a final OD of 10. Volumes of 18 ml of this heavy cell suspension were dispensed in 300 ml Erlenmeyer flasks, and incubated at 36 C with shaking at 130 rotations/min for 2.5 hr. Then the culture was centrifuged at 12,000×*g* for 20 min to remove cells and solid ammonium sulfate was added to the resulting supernatant to a final saturation of 70%, keeping the pH at 7.0 with dilute aqueous ammonia. The mixture was stood at 4 C for about 40 hr, and then the precipitate was collected by centrifugation and dialyzed against physiological saline containing 0.001 M Tris-HCl (pH 7.4) at 4 C until free of sulfate. This concentrated solution of the extracellular protein gave a single major band of protein on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 1) and was used as starting material for purification of β-NG2 protein.

FIGURE 1. *SDS-polyacrylamide gel electrophoresis of the extracellular protein product of C7(β-NG2) and C7(β). (a), C7(β-NG2); (b), C7(β); (c), standard proteins, (1) bovine serum albumin, (2) pepsin and (3) egg white lysozyme. Approximately 50 μg of each protein were applied to gels.*

2. *Preparation of DTT-treated diphtheria toxin*

Partially purified diphtheria toxin, obtained from the Vaccine Production Plant of our Institute (Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa) was purified by two cycles of zone electrophoresis, as described by Katsura et al. (1957). The resulting toxin showed a single protein band on analytical disc gel electrophoresis and was crystallized from solution by addition of ammonium sulfate to 55% saturation. The toxin was treated with dithiothreitol (DTT) by the method of Collier and Kandel (1971). A solution of this purified diphtheria toxin (1 mg protein/ml) in 50 mM Tris-HCl buffer (pH 8.2) containing 1 mM EDTA was incubated at 25 C for 1 hr with 100 mM DTT. The DTT-treated toxin was found to be composed mostly of " fragment A" and " fragment B ", judging from its profile on analytical polyacrylamide gel electrophoresis with or without SDS.

3. *Purification of β-NG2 protein, "fragment A" and "fragment B" by polyacrylamide gel electrophoresis*

β-NG2 protein in the extracellular protein fraction produced by phage-mutant lysogen (C7(β-NG2)) and " fragments A and B " in DTT-treated diphtheria toxin were each purified by the method of Fukui et al. (1971) with slight modifications.

Aliquots of 100 μl (80 μg protein) of the concentrated extracelluler protein of C7(β-NG2) or DDT-treated diphtheria toxin were applied with a droplet of glycerol and 1 μl of 0.004% bromphenol blue to the top of 7.5% polyacrylamide gels (7× 0.5 cm). Electrophoresis was then carried out at a constant current (2 mamp per gel) at 4 C for about 2.5 hr until the tracking dye reached 2 mm from the bottom of the gel. Usually 24 gels were employed in each run of electrophoresis. To locate bands of protein, one gel was stained for 5–10 min with a freshly prepared solution of Coomassie brilliant blue (Weber and Osborn, 1969) diluted 20-fold with 7% acetic acid. The regions of the other gels cor-

responding to the objective protein bands were cut out as lengths of about 1.5–2.0 mm. Protein was eluted and collected from these unstained sections of gels as follows: A stacking gel (7.5%) of 1.5 cm height was first prepared in a 10 cm × 0.5 cm column. The unstained sections of gels were piled on top of each other on the stacking gel, with a little Tris-glycine buffer (pH 8.3), to the top of column. The bottom of the column was put into a dialysis sac containing 0.3–0.5 ml of the buffer and then electrophoresis was performed at a constant current (2 mamp per column) at 4 C overnight using Tris-glycine buffer (pH 8.3) as electrode buffer. The yield and purity of the material collected in the dialysis sac was then examined by analyzing its protein content, ADP ribosylation activity and the patterns on analytical gel electrophoresis and Ouchterlony's immunodiffusion. When impurities were detected, the above purification procedure including separation, elution and collection of the objective protein was repeated. Usually one cycle of purification was enough for β-NG2 protein and "fragment A", but at least two cycles were necessary for "fragment B". Purified "fragment B" was still slightly contaminated (a few percent) with "fragment A", judging from the ADP ribosylation activity of the final product. The yields of these purified proteins were 20–30 per cent of the materials applied and the concentrations of the final preparations were 180–400 μg protein/ml, 150–360 μg/ml and 170–680 μg/ml for β-NG2 protein, "fragment A" and "fragment B" respectively, the amounts depending on the amounts of material subjected to electrophoretic separation and elution. These preparations each gave a single band on analytical gel electrophoresis and the mobility of β-NG2 protein in the gel was similar to that of "fragment A" (Fig. 2).

FIGURE 2. *Polyacrylamide gel electrophoresis of purified β-NG2 protein, "fragment A" and "fragment B". (a), β-NG2 protein; (b), "fragment A"; (c), "fragment B". Approximately 25 μg of each protein were applied to gels.*

4. *Tryptic digestion and peptide mapping on cellulose thin layers*

The procedures used were essentially those described by Fukui et al. (1971) with the following modifications.

Purified protein preparations obtained as described above were dialyzed thoroughly against distilled water at 0 C. Then 0.5 M ammonium acetate buffer (pH 8.0) was added to give a final concentration of 50 mM. The protein solutions were then heated at 100 C for 1.5 min and readjusted to pH 8.0 with dilute aqueous ammonia. Trypsin treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone (Worthington Biochemical Corp.), was added to the heat treated preparations to give a final enzyme to substrate ratio of 1/60 and each mixture was incubated at 25 C for 24 hr in the presence of a trace of thymol (cryst., Merck) to prevent bacterial contamination. After digestion, the mixture was centrifuged at 10,000 × *g* for 30 min to remove traces of insoluble materials and the supernatant was kept at −70 C until subjected to thin layer electrophoresis and chromatography. These procedures were carried out as follows.

Volumes of the tryptic digests of up to 125 μl containing 35–50 μg of material were applied using 25 μl microcapillaries (Drumond Scientific Co. Ltd.) to glass plates (20 cm × 20 cm) precoated with cellulose (TLC-plates cellulose precoated (without fluorescence indicator), Merck) as a 3 cm long band origin. Separation of peptides in the first dimension was achieved electrophoretically using buffer system containing glacial acetic acid-99% formic acid-water (85: 25: 1400, by volume). Electrophoresis was carried out at 830 volts for 25 min

at 10 C in a specially designed apparatus. Other procedures for compressing the series of bands separated by electrophoresis into a fine line, for chromatography in the second dimension and for subsequent color development with ninhydrin were as described by Fukui et al. (1971).

5. *Analytical polyacrylamide gel electrophoresis*

Analytical electrophoresis in polyacrylamide gel was performed using 7.5% gel by the method originally described by Davis (1964). Samples were applied to gel (6.5 cm × 0.5 cm or 7 cm × 0.5 cm) with sample gel or with a droplet of glycerol. Electrophoresis was carried out at a constant current (2 mamp per gel) employing Tris-glycine buffer (Tris 6 g and glycine 28.8 g per liter, pH 8.3) as electrode buffer. Gels were stained with 1% amido black solution and destained electrophoretically in 7% acetic acid.

6. *Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis*

SDS-gel electrophoresis was carried out as described by Weber and Osborn (1969) using 10% gel and the standard buffer system. Bovine serum albumin (Armour Pharmaceutical Co.), pepsin (2x crystallized, Worthington Biochem. Co.) and egg white lysozyme (3x crystallized, Sigma Chem. Co.) were used as standard proteins for estimating molecular weight.

7. *Protein determination*

Protein was estimated by the method of Lowry et al. (1951).

8. *Assay of ADP ribosylation activity*

ADP ribosylation activity was assayed by measuring incorporation of radioactive label from NAD-(adenosine)-^{3}H (590 μCi/μmole, New England Nuclear) into material precipitated with trichloroacetic acid in the presence of a partially purified preparation of rabbit reticulocyte transferase II, following the method of Collier and Kandel (1971) with slight modifications (Matsuda et al., 1972).

9. *Immunodiffusion analysis*

Immunodiffusion was carried out at 30 C in Ouchterlony's plates employing 0.75% agarose in 0.01 M phosphate buffer (pH 7.5) containing 0.5 M glycine, 0.14 M NaCl and 0.02% merthiolate.

10. *Antitoxin*

Pepsin-treated horse antitoxin Lot. no. 68 (1300 Lf/ml) was obtained from Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University.

11. *Chemicals*

Dithiothreitol (DTT) was purchased from Nakarai Chemicals, Ltd. Glacial acetic acid (super special grade), 99% formic acid (guranteed reagent) and ninhydrin (for the automatic amino acid analyser) were from Wako Pure Chemical Industries Ltd., and n-butanol, pyridine and acetone (pro analysi) were Merck's reagents.

RESULTS

1. *Immunodiffusion analyses of β-NG2 protein, "fragment A" and "fragment B"*

It was previously shown that the antigenicity of β-NG2 protein was partially identical with that of whole diphtheria toxin (Matsuda et al., 1972). To clarify the antigenic specificity of the mutant-protein further, β-NG2 protein, "fragment A" and "fragment B" were subjected to immunodiffusion analysis. The results are shown in Figs. 3(a) and 3(b).

Fig. 3(a) shows that β-NG2 protein and "fragment A" each formed a single precipitation band and these bands fused completely with each other, while the single precipitation band formed by a crystalline diphtheria toxin preparation fused with the band of each of these proteins with clear spur formation. On the other hand, the single precipitation band formed by "fragment B", which partly fused with the band of crystalline toxin, clearly crossed that formed by β-NG2 protein (Fig. 3(b)). A similar immunodiffusion pattern to that shown in Fig. 3(b) was obtained when β-NG2 protein was replaced by "fragment A". Heating of β-NG2 protein or "fragment A" at 100 C for 10 min in Tris-glycine buffer (pH 8.3) did not diminish its precipitability with horse antitoxin, but no precipitation band was observable when the preparation of "fragment B" had been heated at

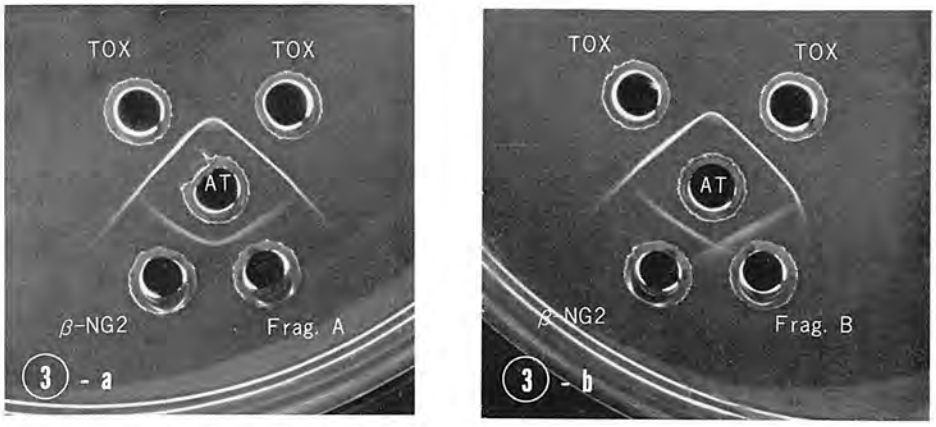


FIGURE 3. *Antigenic relation between β-NG2 protein and "fragments A and B". (a) β-NG2 protein and "fragment A", (b) β-NG2 protein and "fragment B". TOX, 200 Lf units/ml of crystalline diphtheria toxin; AT, 200 units/ml of horse antitoxin; β-NG2, β-NG2 protein 100 μg/ml; Frag. A, "fragment A" 100 μg/ml; Frag. B, "fragment B" 150 μg/ml.*

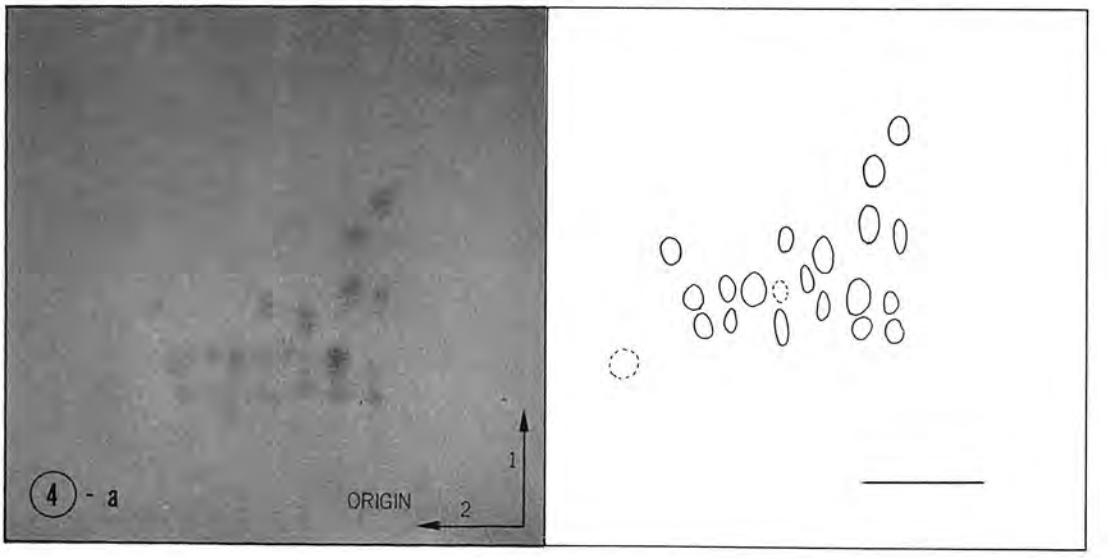
60 C for 10 min.

These results indicate that β–NG2 protein possesses the parts of the antigenic determinants of diphtheria toxin which appear common to those of "fragment A".

2. *Peptide mapping of β–NG2 protein, "fragment A" and "fragment B".*

Purified β–NG2 protein, "fragment A" and "fragment B" were subjected to finger printing by two-dimensional separation of the tryptic digests by electrophoresis and chromatography as described in MATERIALS AND METHODS The resulting peptide maps of these proteins are shown in Figs. 4(a)-(c).

The peptide map of β–NG2 protein is similar to that of "fragment A" but not that of



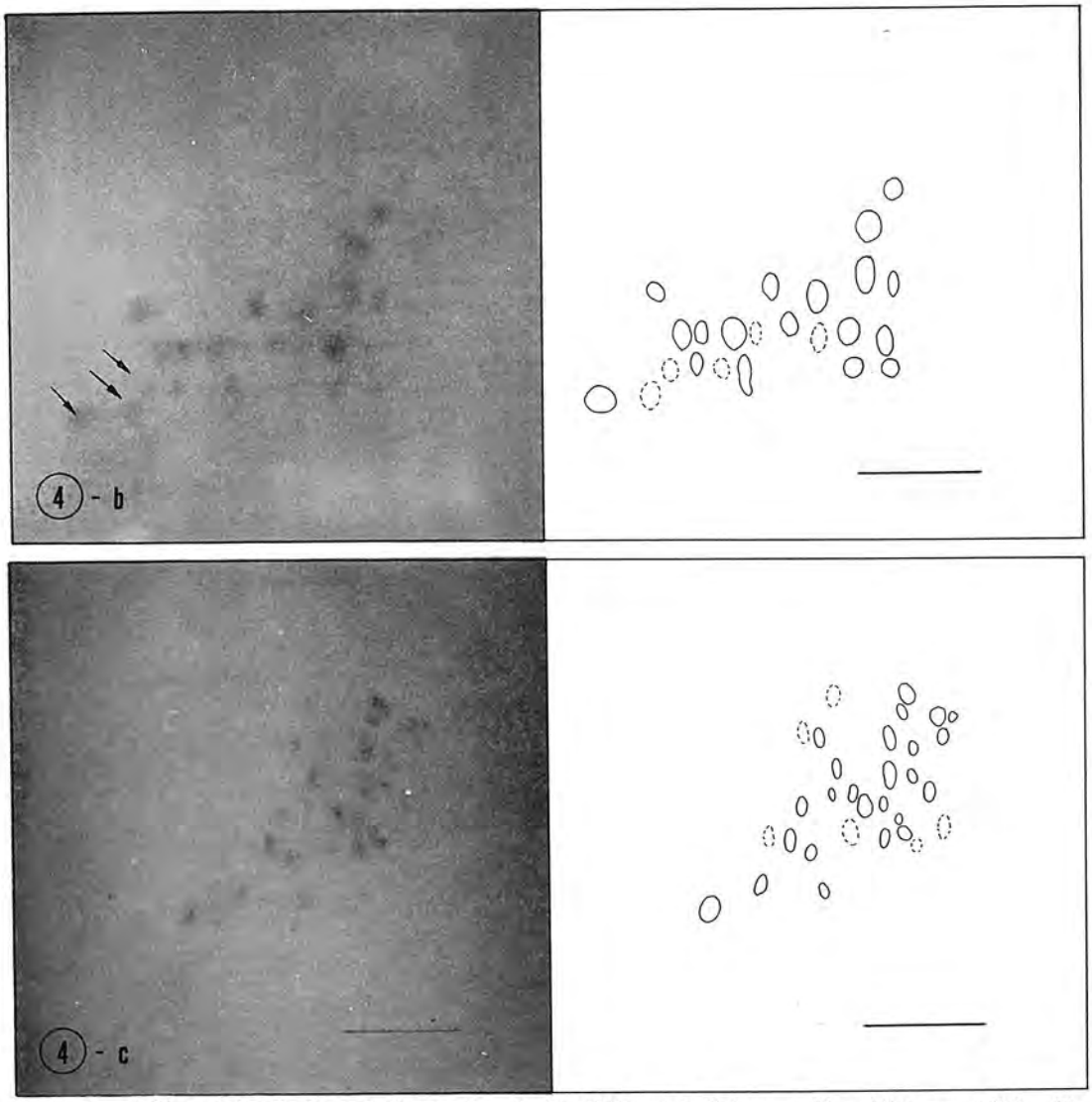


FIGURE 4. *" Finger prints " of tryptic digests of purified β-NG2 protein, " frament A" and " fragment B ". The direction of electrophoresis is indicated by ' 1 ' and that of chromatography by ' 2 ' and all plates were developed with ninhydrin. The digest was prepared from approx. 50 μg of β-NG2 protein and " fragment A" and 35 μg of protein of " fragment B ". (a), β-NG2 protein; (b), " fragment A"; (c), " fragment B ". Arrows in (b) indicate the presence of faint peptide spots which appeared to be absent in (a).*

" fragment B ". The maps of β–NG2 protein (Fig. 4(a)) and " fragment A " (Fig. 4(b)) are composed of about 20 peptide spots and the distribution patterns of their peptides appear to be almost identical in the locations of spots and their relative intensities of staining with ninhydrin. Careful inspection revealed, however, that the peptide map of " fragment A " contains 2 or 3 faint, additional spots in the lower-left corner. The peptide map of " fragment B " (Fig. 4(c)) is composed of more than 30 peptide spots and their distribution

pattern differs greatly from that of β–NG2 protein or " fragment A ".

DISCUSSION

Previously we showed that a single major protein species with a molecular weight of 24,000 (β–NG2 protein) produced by a phage-mutant lysogen (C7 (β–NG2)) has no toxicity but has ADP ribosylation activity and cross-reactivity with diphtheria antitoxin. We suggested that β–NG2 protein resembles " fragment A " of diphtheria toxin (Matsuda et al., 1972). The results presented in this paper provide further evidence of the similarity between these proteins related to the toxin. Thus β–NG2 protein and " fragment A " were shown to have an almost identical peptide " finger print " and antigenic specificity.

Peptide maps of tryptic digests of β–NG2 protein, " fragment A " and " fragment B " revealed 21, 22–23 and more than 30 peptide spots, respectively. The numbers of peptide spots of " fragments A and B " appear to correspond fairly well to those expected from the numbers of lysine and arginine residues in diphtheria toxin (Raynaud et al., 1965 ; Michel et al., 1972) and in the fragments (Michel et al., 1972), suggesting that the tryptic digestion was almost complete. The finding that β–NG2 protein gave 2–3 fewer peptide spots than " fragment A " was reproducible and this suggests that β–NG2 protein is a little bit shorter polypeptide than " fragment A ", provided the latter preparation contains no contaminants.

Immunodiffusion analysis showed that β–NG2 protein has a similar serological specificity to " fragment A ". This implies that the determinant groups of these proteins responsible for combining with precipitating antibody are essentially the same. However, this requires confirmation using anti β–NG2 protein and anti " fragment A " sera. Results also showed a distinct difference in the serological specificities of " fragment B " and β–NG2 protein or " fragment A ", indicating the presence of at least two kinds of precipitating antibody relevant to diphtheria toxin in the horse antitoxin employed (Pappenheimer et al., 1972).

The crude concentrated extracellular product of the lysogen showed no definite protein bands other than that of β–NG2 protein on SDS-gel electrophoresis (Fig. 1) and even a freshly prepared culture supernatant of the lysogen gave a single band of β–NG2 protein on gel electrophoresis. Thus it appears that β–NG2 protein may be produced by C7 (β–NG2) in the form in which it is synthesized. At least it may not be a degradation product of diphtheria toxin or of a fragment of the toxin larger than β–NG2 protein formed by proteolytic enzyme(s) during the isolation procedure.

Recently, Michel et al. (1972) examined the amino acid composition and end groups of diphtheria toxin and of its thiol-splitted sub units (the " L and H chains ") and established that the " L chain " (corresponding to " fragment A ") is the N-terminal moiety of the toxin polypeptide, as suggested by Gill and Dinius (1971). The N-terminal amino acid of β–NG2 protein has not yet been determined. However, β–NG2 protein is in many respects similar to " fragment A ", so it may also be the N-terminal portion of diphtheria toxin, as in the case of CRM45 (mol. weight, 45,000) reported by Uchida et al. (1971).

Previously we showed that production of β–NG2 protein was inhibited by excess iron in the medium and suggested that the N-terminal portion of the toxin might be involved in the mechanism of inhibition of diphtheria toxin by iron. To study this, we are now trying to isolate phage mutants, the lysogen of which may produce much smaller fragment of the toxin than β–NG2 protein.

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