Reconstitution of Diphtheria Toxin from Two Nontoxic Cross-Reacting Mutant Proteins

Abstract. *The isolation of a new type of mutant Corynephage β, which carries a missense mutation in the structural gene for diphtheria toxin synthesis is described. The lysogenic* $C7_s(\beta_{197})^{tox-crm+}$ *strain of* Corynebacterium diphtheriae *produces a nontoxic, extracellular protein of molecular weight 62,000. This protein is immunologically indistinguishable from toxin itself but inhibits the action of toxin on HeLa cells, probably by competing for attachment sites on the cell membrane. In contrast to fragment A derived from diphtheria toxin, fragment* A_{197} *is unable to catalyze the inactivation of eucaryotic polypeptidyl–transfer RNA–transferase II. When mixtures of the two nontoxic mutant proteins, enzymically active* crm_{45} *protein and enzymically inactive* crm_{197} *protein, are subjected to mild treatment with trypsin in the presence of a thiol and then allowed to reoxidize after dialysis to remove excess thiol, "diphtheria toxin" is reconstituted in high yield.*

Diphtheria toxin is synthesized as a single polypeptide chain of about 62,000 daltons by *Corynebacterium diphtheriae* lysogenic for the temperature phage β^{tox+}. When purified toxin is treated with trypsin in the presence of a thiol, a single peptide bond is split (nicked), and the two disulfide bonds are reduced to yield two fragments, A (24,000 daltons) and B (38,000 daltons) (*1*); both fragments are required for toxicity (*2*). In the presence of nicotinamide adenine dinucleotide (NAD), fragment A catalyzes the adenosine diphosphate (ADP)–ribosylation and inactivation of soluble eucaryotic polypeptidyl–transfer RNA–transferase II (translocase) (*3*). It has been suggested that the COOH-terminal fragment B portion of the toxin molecule is required for attachment to and penetration of the membrane of the sensitive cell (*4, 5*). Uchida *et al.* (*5*) have reported the isolation of a *tox*− mutant phage, β_{45}. After lysogenization of the sensitive $C7_s(-)^{tox-}$ strain with β_{45}, the converted $C7_s(\beta_{45})^{tox-crm+}$ strain produces a nontoxic, extracellular protein, crm_{45} (cross-reacting material), of molecular weight 45,000, that is enzymically active and cross-reacts with diphtheria antitoxin. After mild treatment or crm_{45} with trypsin in the presence of thiol, a 24,000-dalton fragment, indistinguishable from fragment A, is formed. The protein crm_{45} is nontoxic because its fragment A fails to reach the sensitive cell interior.

We have since isolated several other mutant strains of β, each carrying an altered *tox* structural gene. Lysogenic C7 strains derived from certain of these mutant phages release cross-reacting proteins that are of reduced toxicity because of alterations that affect enzyme activity in the NH_2-terminal fragment A portion of the molecule. We now report on the properties of an extracellular protein released by $C7_s(\beta_{197})^{tox-crm+}$ which, though completely nontoxic, appears to be immunologically identical with intact toxin. Fragment A_{197} derived from crm_{197} has no enzyme activity, although the intact protein crm_{197} can compete with diphtheria toxin for attachment sites on the HeLa cell membrane. We show that by suitable treatment of mixtures of crm_{45} (lacking the terminal sequence of fragment B, 17,000 daltons) and crm_{197} (with defective fragment A), a fully active toxic protein can be reconstituted in good yield.

Phage mutants were isolated by a modification of the method used to isolate β_{45} (*5*). Nitrosoguanidine was added 20 minutes after ultraviolet irradiation of cultures of $C7_s(\beta)$; 3 to 3.5 hours later, the culture filtrates containing the surviving phage (0.6 to 1 percent of the normal burst) were plated on C7(−). Lysogenized resistant colonies were tested for toxinogeny by the rabbit intradermal test. Several liters of the strain $C7_s(\beta_{197})^{tox-crm+}$ isolated in this way were grown under optimal conditions for toxin production by $C7_s(\beta)$. The crm_{197} protein was purified from the culture supernatant by ammonium sulfate precipitation, dialysis, and chromatography on diethylaminoethylcellulose.

Some properties of purified crm_{197} are compared with those of purified crm_{45}, toxin, and toxoid in Table 1. The chemical and physical properties of the crm_{197} protein are very similar to those of diphtheria toxin itself. The two proteins have the same molecular weight of 62,000, as determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Toxin and crm_{197} are immunologically indistinguishable, giving curves that are superposable when tested by quantitative flocculation against a horse antitoxin. The specific toxicity of crm_{197}, however, is less than one-millionth that of diphtheria toxin. As much as 0.5 mg (250 flocculating doses, Lf) of purified crm_{197} injected into guinea pigs failed to produce even a local reaction at the site of injection. Two rabbits were first injected with 0.2 mg of crm_{197} in complete Freund adjuvant; 1 month later, a booster injection of 0.2 mg was given, and a week later 0.4 mg was given. (There were no toxic reactions.) One week afterward the antiserums gave positive ring tests with

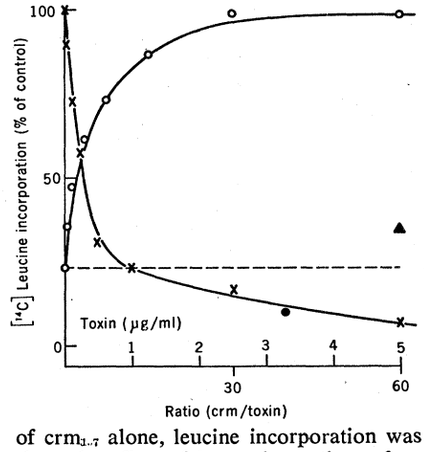

Fig. 1. Competition between crm_{197} and toxin for HeLa cell binding sites. Washed suspensions of growing HeLa cells were suspended in Eagle's medium containing 2 percent fetal calf serum and then distributed in 2-ml amounts in roller tubes containing increasing amounts of purified crm_{197}. After 30 minutes rotation at 5 rev/min at 37°C, diphtheria toxin (1 μg/ml; 0.5 Lf/ml) was added to all tubes, except for certain control tubes. After incubation for 3.5 hours more, 100 μl of [^{14}C]leucine (1 μc/ml) was added to each tube. Cells were harvested 3.5 hours later on Millipore filters, washed first with Hanks salt solution, then with 5 percent trichloroacetic acid, dried, and counted. Leucine uptake by control tubes without toxin was taken as 100 percent. In the presence of 60 μg of crm_{1-7} alone, leucine incorporation was the same as in the control. The open circles show the effect of increasing ratios of crm_{197} to toxin on [^{14}C]leucine uptake plotted as percentage of the control uptake. The crosses represent a titration of toxin under the same experimental conditions. Incorporation of leucine in the presence of toxin (1 μg/ml) alone was 23 percent of the control value (dashed line). The closed circle and triangle show leucine uptake in the presence of crm_{45} and purified toxoid in ratios to toxin of 40 : 1 and 60 : 1, respectively.

Table 1. Some properties of diphtheria toxin and cross-reacting proteins.

Protein	Molecular weight	ADP-ribosylating activity *	Toxin-blocking activity †	Toxicity ‡ (%)	Antitoxin precipitated§
Toxin	62,000	100	—	100	100
crm_{45}	45,000	100	0	<0.0001	70
crm_{197}	62,000	0	+	<0.0001	100
Toxoid	62,000	0	0	0	100
Fragment A	24,000	100	0	<0.0001	25

*Expressed as percent of activity of fully activated toxin on a molar basis. †Ability to block reversibly the inhibition by toxin of amino acid incorporation into HeLa cells. ‡Expressed as percent of intradermal toxicity of purified toxin. § Expressed as percent of antibody precipitable from a horse antitoxin by toxin.

purified toxin and contained 1 and 5 units of neutralizing antitoxin per milliliter by rabbit skin test.

When purified crm_{197} is subjected to mild digestion with trypsin in the presence of dithiothreitol (DTT), it breaks down, as does toxin, into two dissimilar fragments of 24,000 and 38,000 daltons, respectively, which can be separated by SDS–polyacrylamide gel electrophoresis. Unlike toxin, however, neither intact crm_{197} nor fragment A_{197} derived from it shows any enzymic activity. Under the usual test conditions (*2*), as little as 2 ng of *activated* diphtheria toxin catalyze the rapid breakdown of NAD and transfer of its ADP ribose group to eucaryotic translocase. Under identical conditions, 10,000 times as much crm_{197} failed to show detectable ADP-ribosylating activity, whether tested before or after treatment with varying amounts of trypsin in the presence of DTT. The failure of crm_{197} to show toxicity is therefore due to a mutation causing loss of the enzymic activity associated with the fragment A portion of the toxin molecule. It seems likely that the alteration leading to loss of enzymic activity is located in the NAD-binding site. In the first place, crm_{197} protein has no effect on the enzymic activity of nicked toxin in vitro even when the ratio of crm_{197} to toxin exceeds 100 : 1. This result shows that crm_{197} does not interfere with the formation of the ternary complex of fragment A, and NAD, and translocase (*6*). Finally, we have been unable to detect appreciable binding of [^{14}C]NAD even by 1.5 percent solutions of purified crm_{197}, by the technic of equilibrium dialysis. Under the same conditions, binding of NAD by far lower concentrations of toxin is easily measured (*7*).

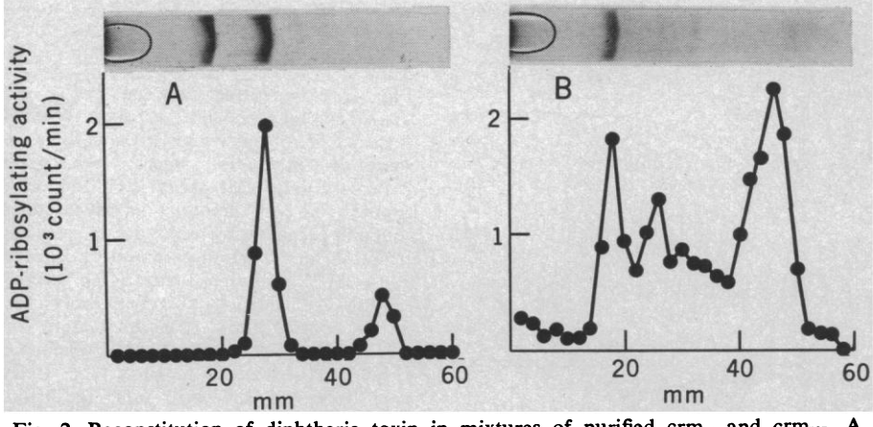

Fig. 2. Reconstitution of diphtheria toxin in mixtures of purified crm_{45} and crm_{197}. A mixture was prepared in 0.05*M* tris at *p*H 8.0 containing crm_{45} (2.4 mg/ml) and crm_{197} (1.6 mg/ml) equivalent to a molar ratio of 2 : 1. A portion of the mixture was treated with crystalline trypsin (5 μg/ml) in the presence of 10 m*M* DTT. After 10 minutes at 37°C, the reaction was stopped by addition of soy bean–trypsin inhibitor. The ADP-ribosylating activity of the mixture was increased about twofold by this treatment. The digestion mixture was then dialyzed in the cold against 0.01*M* phosphate, *p*H 7.2 to remove the excess DTT. Both mixtures, before (A) and after (B) treatment with trypsin, were analyzed in duplicate on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with 10- and 20-μl samples, respectively. No thiol was added to the gels. One of each of the gels was stained (shown at top); the other was analyzed for ADP-ribosylation activity (*2*) after elution from 2-mm slices.

Table 1 also shows that the nontoxic crm_{45} protein, which lacks the 17,000-dalton COOH-terminal amino acid sequence of toxin, has very different properties from those of crm_{197}. Although fully active enzymically, crm_{45} is nontoxic because it cannot be taken up by sensitive cells. It was therefore necessary to find out whether the enzymically inactive crm_{197} still retained the capacity to interact with specific toxin-binding sites on the HeLa cell membrane. Figure 1 shows that this is indeed the case and that crm_{197} can successfully compete with toxin for attachment to HeLa cells and thus block the inhibition by toxin of amino acid incorporation into cell protein. The number of toxin-binding sites on the HeLa cell membrane is small (*8*). From the preliminary data, the affinity of crm_{197} for these sites would not appear to be very different from that of toxin itself. Nevertheless, even a large excess of purified crm_{45} or of purified diphtheria toxoid failed to interfere significantly with the binding of toxin by the cells. We conclude from these experiments that the COOH-terminal, 17,000-dalton portion of fragment B is essential for attachment of toxin to the HeLa cell membrane and that in its absence enzymically active fragment A portion of the molecule cannot gain access to the cytoplasm.

Final and striking confirmation of the above conclusion has come from experiments in which toxin molecules were reassembled from fragments derived from the two nontoxic crm proteins. When purified preparations of crm_{45} and crm_{197}, containing only traces of nicked protein (*1*) were mixed and allowed to remain overnight in the cold, the mixtures became toxic, although the actual amount of toxicity developed was very low. However, when similar mixtures were kept under the same conditions in the presence of 10 m*M* DDT, appreciable toxicity always developed and was equivalent to about 2 to 5 percent of that to be expected if all of the crm_{197} in the mixture had been converted to toxin. It is estimated that about 5 to 10 percent of the crm_{197} in the purified preparation was present in the nicked form. These observations suggested that toxin was being reconstituted from fragments of crm proteins. The yield of reconstituted toxin is greatly increased when the mixture of proteins is treated with trypsin in the presence of DDT (Fig. 2). Before treatment, the nontoxic mixture

gave two major bands on SDS–gel electrophoresis at positions corresponding to proteins of 62,000 daltons (crm_{197}) and 45,000 daltons (crm_{45}) (Fig. 2A). Almost all of the enzymic activity was associated with the crm_{45} peak, but it is evident that a small amount of free fragment A (24,000 daltons) was also present. After removal of DTT and reoxidation, a significant proportion of the activity had moved over into the 62,000-dalton peak (Fig. 2B). In fact, the shift of enzymic activity into this peak corresponds to nearly 25 percent of all the crm_{45} originally added and is equivalent to approximately half of the activity to be expected if all the crm_{197} had been converted into active enzyme. The trypsin-treated dialyzed mixture was injected, intraperitoneally, into guinea pigs (250 to 280 g) in amounts corresponding to 10, 5, and 2.5 μg of the crm_{197} originally present. The survival times were 10 to 12, 14 to 16, and 20 hours, respectively. From the dose-survival curve of Baseman *et al.* (*4*), it may be calculated that the yield of reassembled toxin corresponds to between 40 and 80 percent of the crm_{197} originally added, a result in excellent agreement with the proportion of enzymic activity shifted into the 62,000-dalton peak (*9*). Antitoxin specifically neutralized the toxicity of the reconstituted mixture.

In conclusion, we have demonstrated that each of the two dissimilar fragments comprising the diphtheria toxin molecule has a separate and distinct function. Toxicity depends on the unique enzymic activity associated with fragment A. This activity is responsible for the arrest of polypeptide chain elongation in sensitive cells by specific ADP-ribosylation of translocase. But fragment A can only reach the sensitive cell cytoplasm when it is specifically associated with fragment B. The number of sites on HeLa cells available to react with toxin molecules is small (*8*). Since crm_{197}, but not crm_{45}, successfully competes with toxin in HeLa cell cultures, it seems most probable that attachment and penetration of toxin are specific processes that depend on the composition and conformation of the 17,000-dalton COOH-terminal amino acid sequence of the molecule.

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9. It will be noted from Fig. 2B that, after treatment with trypsin, relatively little material remained in the 45,000-dalton band (despite the fact that crm_{45} was in twofold excess), possibly because fragment A has a higher affinity for intact fragment B derived from crm_{197} than for the corresponding 21,000-dalton fragment derived from crm_{45} or for another fragment A molecule to form a dimer. Because of the relative instability of fragment B, some degradation probably always occurs, and the reconstituted mixture therefore contains an excess of fragment A, seen as a fast-moving component.
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Isozymes of Phenylalanine Hydroxylase

Abstract. *Three isozymes of phenylalanine hydroxylase exist in adult rat liver. They are chromatographically unique. Partial chracterization suggests that they are similar in chemical properties and differ only in charge. Estimation of the Stokes radii indicates that the isozymes have similar molecular weights of about 200,000. Two isozymes exist in human fetal liver. Alterations of the relative amounts of these isozymes may control the phenotype of the disease phenylketonuria.*

Phenylalanine hydroxylase (E.C. 1.14.3.1) catalyzes the irreversible hydroxylation of phenylalanine at the para position to yield tyrosine (*1*). The hydroxylating system consists of several protein components (*2*) and requires reduced nicotinamide adenine dinucleotide phosphate and pteridine as cofactors (*3*). Huzino and Bessman separated the hydroxylase component into two isozymes (*4*). Kaufman and Fisher also found two forms of the enzyme and further characterized them (*5*).

We have separated and characterized three isozymes from adult rat liver and have distinguished between two isozymes from human fetal liver. The three forms isolated from rat liver, which we have called pi, kappa, and upsilon, appear to be three distinct isozymes of phenylalanine hydroxylase and not conformational isomers.

All procedures were carried out at 4°C unless otherwise noted. Male rats (Sprague-Dawley strain), 150 to 250 g, were decapitated, and the livers were homogenized in two volumes of buffer that contained 0.025*M* K_2HPO_4, 0.15*M* KCl, and 5 m*M* dithiothreitol (DTT), *p*H 6.8. The homogenates were centrifuged at 100,000*g* for 30 minutes, and a volume of clear supernatant containing 30 mg of protein (estimated by the biuret method) was placed on a column of neutral calcium phosphate gel and wet cellulose powder (30:70, by volume). Column dimensions were 7 by 200 mm and bed volume was 10 ml. The sample was eluted with a linear gradient of K_2HPO_4, 0.025 to 0.2*M*, *p*H 6.8. The gradient also contained 0.15*M* KCl and 5 m*M* DTT; total volume was 50 ml. Flow rate was approximately 0.25 ml/min, and all of the recoverable activity was eluted in 100 0.4-ml fractions. The yield was 90 to 100 percent.

The specific activity of the most active fraction of the kappa peak was about tenfold greater than the specific activity of the crude supernatant.

Table 1. Properties of isozymes of phenylalanine hydroxylase; K_m is the Michaelis constant. Thermal inactivation (therm. inact.) is the percentage of enzyme inactivated by 8 minutes at 42°C. The inhibition (Inhib.) study used 10^{-3}*M* D,L-*p*-chlorophenylalanine (*p*-CPA).

K_m (mmole of tyrosine)	Optimum *p*H	Therm. inact. (%)	Inhib. by *p*-CPA (%)
	Pi		
0.87	7.2	40.0	43.1
	Kappa		
0.78	7.2	26.1	40.2
	Upsilon		
0.64	7.2	47.4	51.4