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PREPARATION AND PROPERTIES OF IMMOBILIZED DIPHTHERIA TOXIN

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Commercially available diphtheria toxin was immobilized on nylon stocking material by a covalent activation of the polymeric material. Toxin was bound both directly to nylon as well as to nylon-polyethyleneimine copolymer. The immobilized toxin retained some of its catalytic activity as evidenced by ADP-ribosylation of elongation factor II. Kinetics of this reaction are reported. After repeated use for several times the immobilized toxin retains 75% of its activity.

INTRODUCTION

Diphtheria toxin secreted by the bacterium *Corynebacterium diphtheriae* is a zymogen of molecular weight 63,000 (1). It inhibits protein biosynthesis in eucaryotic cells. The zymogen splits into two fragments, A and B, by proteolysis, followed by a reduction of a disulfide bridge that links the two fragments (2), before it can exert its toxicity (Fig. 1).

In order that it can exert its lethal activity, it is believed that fragment A (mol. wt. 24,000), which represents the *N*-terminal fragment, must traverse across the cell membrane. This hydrophilic polypeptide fragment chain interacts with NAD and transfers its ADP-ribose moiety to the eucaryotic elongation factor (EF) II. This inactivates EF II and prevents its further participation in protein biosynthesis. However, with *in vitro* systems the toxin inactivates EF II by producing the dead-end complex ADPR-EF II (3).

The process by which the toxic fragment A reaches the cytoplasm requires the presence of fragment B (mol. wt. 39,000) of the toxin (3). The

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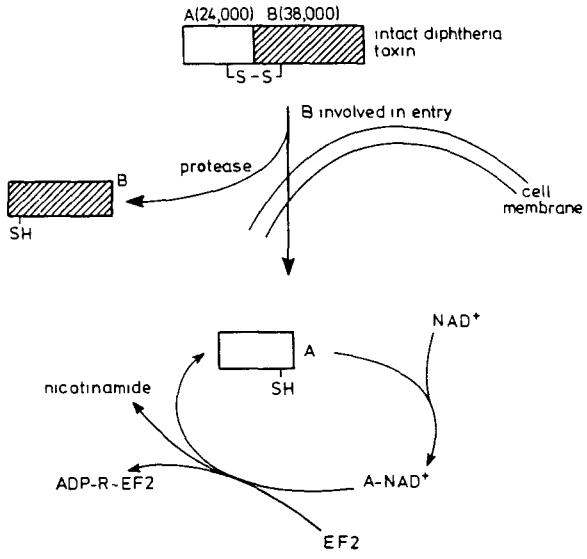

FIG. 1. A stylized depiction of the mode of action of diphtheria toxin on whole cells.

entry of fragment A is preceded by the binding of fragment B to a hitherto unknown receptor molecule on the cell membrane. Bocquet et al. (4) recently proposed that the hydrophobic fragment B binds to the cell membrane and opens up a channel across the lipid bilayer, enabling the hydrophilic fragment A to cross the membrane. More recently, Bocquet (5) proposes that fragment B has two distinct structural regions: one is specific for binding to the cell, while the other more hydrophobic region inserts itself into the lipid layer.

The experiments reported herein were devised at a time when the precise sequence of events leading to the inhibition of protein biosynthesis in whole cells was not known. If upon immobilization the toxin could still inhibit protein biosynthesis in whole cells, then it would suggest that existing concepts of its mode of action might have to be revised. Also, from the evolutionary point of view it is important to understand how a mode of action such as that of the diphtheria and cholera toxins, which are produced by single-cell organisms, is still retained by plant toxins such as ricin and abrin, higher up on the evolutionary ladder.

MATERIALS AND METHODS

Diphtheria toxin isolated from *C. diphtheriae* was kindly supplied by Behring Werke GmbH. EF II was isolated from rat liver according to the

method described previously for human tonsils (6). [^{14}C]-labeled NAD at 274 Ci/mol was obtained from the Radiochemical Center, Amersham, U.K. The assay method to detect toxin activity consisted of incubating at 20°C 2–5 μM [^{14}C] NAD, 1.52 μM EF II and 1–2 μg whole toxin, 0.05 M pH 7.4 Tris buffer, and 0.25 M sucrose containing 10 μM 2-mercaptoethanol for 2–12 min. This follows the method of Bermek (7).

Immobilization of Toxin onto Nylon Strips

Commercially available nylon stocking material was cut to 2×2 cm strips and immersed in about 2 mL of dimethyl sulfate in a flask closed with a ground glass stopper, which was immersed in a boiling water bath and swirled for about 40 sec. The reaction was stopped by transferring the flask to a melting ice bath. The reacted nylon strips were washed with ice-cold methanol several times followed by ice-cold water. The wash solution turns cloudy due to dissolved nylon. The strips, free of this dissolved nylon, were carefully removed by thorough washing.

Protein can be coupled directly to the nylon alkylated in this manner in pH 7.4 Tris buffer containing 10 μM mercaptoethanol or by means of a spacer molecule such as polyethyleneimine (PEI) molecule and cross-

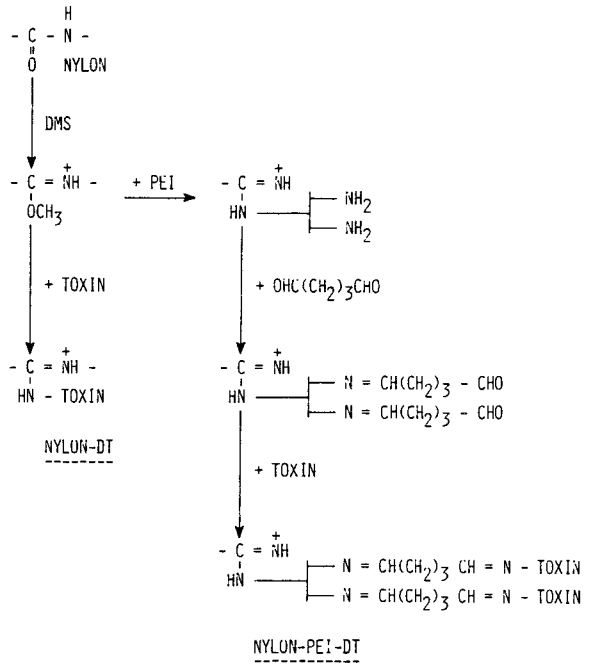

FIG. 2. Methods of coupling diphtheria toxin to nylon and nylon-PEI copolymer.

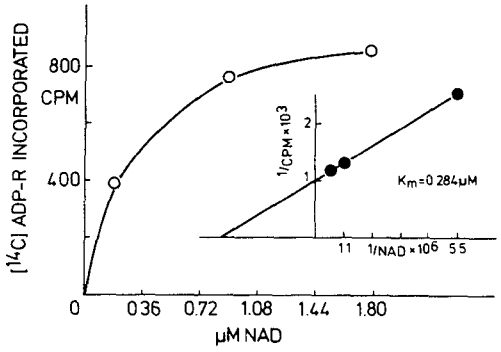

FIG. 3. Kinetics of ADP-ribosylation as a function of [NAD] with nylon-DT.

linking with glutaraldehyde at pH 7 by the method of Sundaram (8.9). With the second method, PEI was coupled as a 1% v/v solution in pH 7.8 phosphate buffer (Fig. 2) made from commercial solution supplied by Serva GmbH.

RESULTS AND DISCUSSION

It was observed that toxin coupling to alkylated nylon is an extremely rapid process, with up to 90% coupling within the first 10 min. It was found that the diphtheria toxin coupled either directly to alkylated nylon (nylon-DT) or to nylon-PEI copolymer (nylon-PEI-DT) retained its catalytic activity as shown by its capacity to ADP-ribosylate EF II in an *in vitro* system.

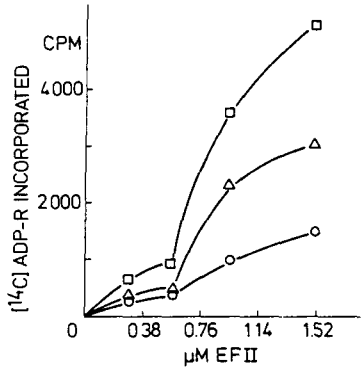

FIG. 4. Kinetics of ADP-ribosylation as a function of [EF II] with nylon-DT. ○, △, □ are 4-, 8-, and 12-min end-point assays.

TABLE 1. **Michaelis Constants of Free and Immobilized Diphtheria Toxin in ADP-ribosylation of EF II**[a]

	Free toxin (μM)	Nylon-toxin (μM)	Nylon-PEI-toxin (μM)
NAD	0.15	0.284	
EF II	0.71		
Low [EF II]		0.43	0.56
High [EF II]		1.15	2.0

[a]Counting efficiency for [^{14}C] was 60% in all the experiments.

The kinetics of this process of ADP ribosylation was studied. Figure 3 shows the effect of varying the concentration of NAD with nylon-DT, keeping the concentration of EF II constant. This led to a typical Michaelis–Menten type plot. However, when EF II concentration was varied in the assay, keeping NAD concentration constant, a distinct biphasic plot was obtained, typified by a sluggish initial part up to 0.52 μM EF II concentration, followed by a burst at higher concentrations (Fig. 4). End-point assays of varying lengths of time, from 4 to 12 min, displayed the same type of behavior. Naturally double reciprocal plots of these data lead to a possibility of obtaining several values for apparent K_m depending upon which region of the curve above or below this critical point of 0.52 μM is extrapolated back to obtain these values (Table 1). Table 1 also shows values of K_m and apparent K_m for NAD.

Toxin immobilized by cross-linking upon nylon-PEI copolymer also catalyzed the transfer of the ADP-ribosyl moiety to EF II. However, the reaction showed a pronounced initial lag. Even though enough toxin may be

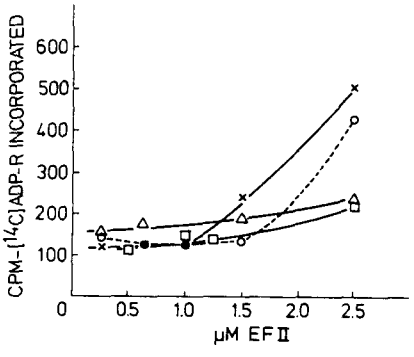

FIG. 5. Kinetics of ADP-ribosylation as a function of [EF II] with nylon-PEI-DT. □, △, ○, ×, are 1.5-, 4-, 8-, and 12-min end-point assays. Note the [EF II]-dependent and time-dependent initial lag of the reactions.

coupled to the polymer by this method, increased points of its attachment on the PEI spacer molecule may be instrumental in reducing the specific activity of the toxin. Besides, diffusion effects may be accentuated by electrostatic interactions caused by the positively charged nylon-PEI derivative.

End-point assays of varying lengths of time ranging from 1.5 to 12 min (Fig. 5) showed that this initial lag is dependent upon the concentration of EF II and the length of time the assay medium is in contact with the nylon-PEI-DT. In other words a 12-min assay shows a shorter lag and a greater transfer of [^{14}C] ADPR to EF II. Slightly higher apparent K_m values for EF II in the case of nylon-PEI-DT compared to nylon-DT is consistent with the above observations.

To establish that the immobilized toxin displayed the catalytic activity, the nylon-DT was used repeatedly, and as shown in Fig. 6, the activity diminished by about 25% after about 25 times, each point in Fig. 6 being an average of six assays. However, the repeated usability, plus the fact that the concentrated supernatant, produced by washing the preparation with buffer after filtering with a Minicon filter, showed no appreciable catalytic activity when tested with the same assay system, confirms that nylon-DT is active.

Honjo et al. (10) reported observing an activation of the catalytic activity of the toxin upon exposure to temperatures above 60°C. Figure 7 shows the results of such an experiment wherein the free toxin does show activation when exposed to various temperatures up to 80°C for 5 min at pH 7.4. However, the immobilized toxin does not exhibit this property, which is probably diminished upon immobilization due to a restricted freedom of the protein molecule.

The main feature of this study is that the immobilized toxin retains its catalytic activity with *in vitro* systems as shown by its ability to transfer the ADP-ribosyl moiety to EF II. Coupling of the toxin in the presence or

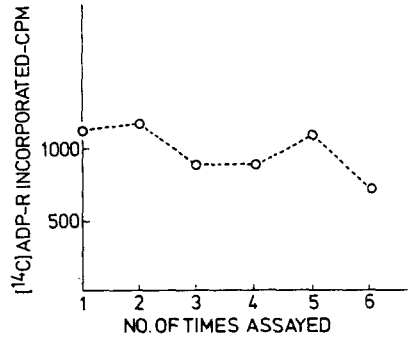

FIG. 6. Effect of repeated use of immobilized toxin (nylon-DT) upon activity.

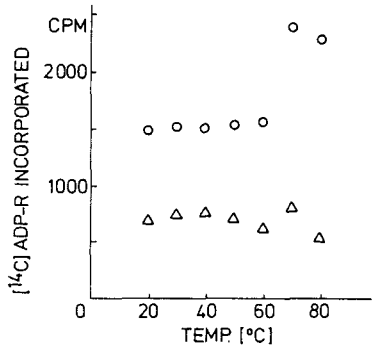

FIG. 7. Effect of incubation at various temperatures prior to assay of free and immobilized toxin (nylon-DT). ○, free diphtheria toxin; △, nylon-DT.

absence of 2-mercaptoethanol did not affect its catalytic properties in the *in vitro* system. Even though with *in vitro* assays this does not have special significance, a similar finding against whole cells may have specific implications, since fragments A and B come apart due to a reduction by mercaptoethanol of the disulfide bridge that links them.

It is essential to demonstrate retention of catalytic activity against *in vitro* systems before further investigation with whole cells. This should be able to answer other interesting questions, such as whether the toxin can inhibit protein biosynthesis and thereby cause protein lysis by mediating through the cell membrane or whether the toxin must actually enter the cell before it can be active.

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REFERENCES

1. COLLIER, R. J., and KANDAL, J. (1971) J. Biol. Chem. 246 : 1496–1503.
2. GILL, D. M., and PAPPENHEIMER, A. M. (1971) J. Biol. Chem. 246 : 1492–1495.
3. COLLIER, R. J. (1975) Bact. Rev. 39 : 54–85.
4. BOCQUET, P., SILVERMANN, M. S., PAPPENHEIMER, A. M., Jr., and VERNON, B. W. (1976) Proc. Nat. Acad. Sci. U.S.A. 73 : 4449–4453.
5. BOCQUET, P. (1977) Biochem. Biophys. Res. Commun. 75 : 696–702.

6. BERMEK, E., and MATTHAEI, M. (1971) Biochemistry 10 : 4906–4912.
7. BERMEK, E. (1972) FEBS Lett. 23 : 95–99.
8. SUNDARAM, P. V. (1974) Nucleic Acids Res. 1 : 1587–1599.
9. SUNDARAM, P. V. (1977) In Biomedical Applications of Immobilized Enzymes and Proteins, Vol. 2 CHANG, T. M. S. (ed.), Plenum, New York, pp. 317–340.
10. HONJO, T., NISHIZUKA, Y., KATO, I., and HAYAISHI, O. (1971) J. Biol. Chem. 246 : 4251–4260.