

Diphtheria toxin: Nucleotide binding and toxin heterogeneity

(flow dialysis/receptor binding/polyphosphate/affinity chromatography)

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ABSTRACT We have used flow dialysis to demonstrate binding of ATP and related compounds to diphtheria toxin. The results define a new site on the toxin molecule (the P site), which has distinctly different properties from the NAD⁺-binding site of the fragment A moiety. The relative affinities of various compounds for the P site are similar to their capacities to inhibit toxin attachment to cell surfaces and its action on cells. This suggests that the P site may correspond to the binding site for cell surface receptors. Affinity of nucleotides for the toxin depends strongly on the number of phosphates, although both nucleoside and phosphate moieties contribute to the interaction. A substantial fraction of the toxin in any given preparation did not bind ATP in a rapidly reversible manner and was not retained on ATP-Sepharose. This fraction, which varied in magnitude from preparation to preparation, was isolated and shown to contain an endogenous, firmly bound nucleotide or nucleotide-like compound. The presence of this compound may explain some of the physical heterogeneity within individual preparations of purified toxin as well as variations in physical and biological properties among various preparations.

Diphtheria toxin kills susceptible mammalian cells by a sequence of events beginning with its binding to receptors on the cell surface (1, 2). The toxin, or at least its catalytic center, then somehow traverses the plasma membrane and becomes exposed to the cytosol. There it inactivates elongation factor 2 by catalyzing covalent attachment of the ADP-ribosyl moiety of NAD⁺. This, in turn, causes inhibition of protein synthesis and cell death.

Middlebrook and coworkers (3, 4) have shown that ATP and certain related phosphate-containing compounds inhibit the action of diphtheria toxin on whole cells and that this inhibition results from interference with toxin attachment to the cell surface. Also, Chang and Neville (5) demonstrated inhibition by ATP or β,γ -methylene ATP of toxin binding to isolated surface membranes from various animal sources. The simplest explanations for these findings would be: (i) ATP binds to the toxin and blocks its receptor-binding site or (ii) ATP binds to the cell surface and alters the toxin-binding capacity of the receptor. The results reported here strongly suggest that the former explanation is correct. In addition, they reveal a previously undisclosed source of heterogeneity of diphtheria toxin, correlated with the presence of a low molecular weight, 260-nm-absorbing compound firmly bound to a fraction of the toxin molecules.

MATERIALS AND METHODS

Flow dialysis was performed by the method of Colowick and Womack (6). The two chambers (upper chamber, 0.6 ml; lower chamber, 0.2 ml) were separated by Spectropore 2 dialysis membrane (Spectrum Medical Industries, Los Angeles). To the upper chamber, containing diphtheria toxin or other protein

(0.25 ml, 30–175 μ M protein) in 50 mM Tris-HCl (pH 7.2), was added 10 μ l of [γ -³²P]ATP (final concentration 0.1 μ M; specific radioactivity 220–110 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), and the same buffer was pumped through the lower chamber at about 3 ml/min. Fractions of 52 drops each were collected and radioactivity was determined by Cerenkov radiation in a Beckman LS-100C liquid scintillation counter. Unlabeled ATP was added in 1- μ l portions every fifth fraction. When inhibition of ATP binding was to be measured, the compound tested was added before the [³²P]ATP. All data were corrected for loss of label from the upper chamber.

ATP-Sepharose (ATP- ϵ -aminohexanoyl-Sepharose 4B) was prepared by coupling ATP to hexanoic acid-Sepharose 4B by a method described for preparation of NAD⁺- ϵ -aminohexanoyl-Sepharose 4B (7). Affinity chromatography of diphtheria toxin was performed in 50 mM Tris-HCl (pH 7.2). After application of the sample, the column was washed with the same buffer and adsorbed toxin was eluted with 0.5 M NaCl in Tris buffer.

[³²P]ATP labeled in the γ -phosphate was obtained from ICN. Nucleotides, polyphosphates, and inositol hexaphosphate were from Sigma. Urea was the Ultrapure grade from Schwarz/Mann.

Diphtheria toxin was purchased from Connaught Laboratories (Toronto, Canada). Its further purification and the preparation of fragment A have been described (8). CRM45 was prepared by a method to be described elsewhere (unpublished data). Briefly, the procedure involved the following steps: precipitation of protein from culture fluids with zinc acetate, ammonium sulfate precipitation of the redissolved protein, chromatography on DEAE-Sephacel (Pharmacia), and gel filtration on Sephacryl S-200 (Pharmacia).

RESULTS

Binding of ATP to diphtheria toxin was demonstrated by flow dialysis with [γ -³²P]ATP. Results of a typical experiment are shown in Fig. 1. From a Scatchard plot of the data we determined the K_d of ATP to be 18 μ M and n , the average number of binding sites per molecule, to be 0.4. Among various preparations of toxin tested, the value of K_d was approximately constant (18–20 μ M), whereas variation was observed in values of n (0.28, 0.32, and 0.4, for three preparations). Both K_d and n were reproducible for a given preparation of toxin. No change in either value was observed when a preparation containing over 90% of the toxin molecules in the intact form was completely nicked with trypsin. We confirmed binding of ATP to toxin by the method of Hummel and Dreyer (9) with [*adenine*-³H]ATP (data not shown).

When whole toxin was replaced by fragment A, a proteolytic fragment of M_r 21,000 that carries the catalytic center of the toxin, no binding of ATP was observed. This finding agrees with the studies of Kandel *et al.* (10), in which it was shown that ATP had little affinity for the NAD⁺-binding site on fragment A ($K_d \gg 180 \mu$ M) and was a poor competitive inhibitor of the ADP-

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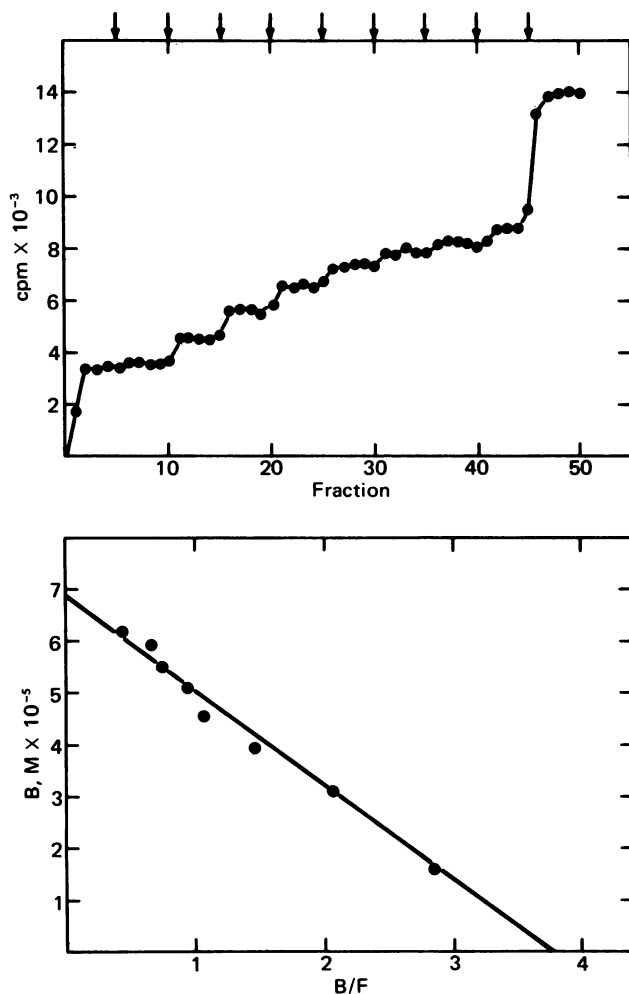


FIG. 1. Measurement of binding of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to diphtheria toxin by flow dialysis. (Upper) $[\text{}^{32}\text{P}]\text{ATP}$ ($0.14\ \mu\text{M}$) was added to diphtheria toxin ($0.26\ \text{ml}$, $175\ \mu\text{M}$) in the upper chamber of the dialysis apparatus and, at intervals, as indicated by the arrows, $1\text{-}\mu\text{l}$ additions of $5.74\ \text{mM}$ unlabeled ATP were made. The final addition was $3\ \mu\text{l}$ of $0.15\ \text{M}$ ATP. (Lower) Scatchard plot of data from the experiment illustrated in the Upper panel. From the slope, the K_d was $18.1\ \mu\text{M}$; from the ordinate intercept, the number of binding sites was 0.4 per mole of toxin. B, bound; F, free.

ribosyltransferase reaction ($K_i \approx 3000\ \mu\text{M}$). Similarly, no binding of ATP was observed with any of the following: CRM45, an enzymically active M_r 45,000 fragment of the toxin; exotoxin A from *Pseudomonas aeruginosa*; or bovine serum albumin. The second of these has the same intracellular mode of action as diphtheria toxin but is believed to bind to a different cell surface receptor (11).

Competitive binding of other compounds to the ATP-binding site on diphtheria toxin was measured by flow dialysis in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and unlabeled competitor (Table 1). Little difference in affinity was observed with variation of the purine or pyrimidine moiety of nucleoside triphosphates, but affinity showed a strong dependence on the phosphate content of the ligand. Adenosine tetraphosphate had a higher affinity than ATP, ADP and AMP had progressively lower affinities, and adenosine or adenine bound only weakly. A nonhydrolyzable analog of ATP, β,γ -methylene ATP, bound to the toxin with affinity comparable to that of ATP. Phosphate-containing compounds lacking a nucleoside moiety, including polyphosphates and inositol hexaphosphate, showed significant affinity for the ATP site. Polyphosphates exhibited a progression in

Table 1. Competitive inhibition of ATP binding to diphtheria toxin by various compounds

Compound tested	K_i^* , μM	Conc. giving 50% inhibition of toxin binding,† μM
Adenosine 5'-tetraphosphate	4	20
ATP	18	100
ADP	29	250
AMP	221	2000
Adenosine	>1000	ND
Adenine	>1000	ND
GTP	18	20
ITP	19	—
UTP	21	200
β,γ -Methylene ATP	15	—
Tetrapolyphosphate	12	—
Tripolyphosphate	36	1000
Pyrophosphate	1700	—
Orthophosphate	3700	—
Trimetaphosphate	35	—
Inositol hexaphosphate	60	—

* Flow dialysis measurements were performed as described for Fig. 1, except that the compound tested was added before $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Values of K_i were calculated from reciprocal plots. Inhibition was competitive in all cases.

† From data of Middlebrook and coworkers (3, 4). The numbers represent the concentration required to inhibit by 50% the binding of ^{125}I -labeled diphtheria toxin to Vero cells during a 12-hr incubation at 4°C . ND, not detectable at the highest concentration tested; —, either not tested or numbers could not be derived from the data presented.

affinity with increasing chain length similar to that found with adenine nucleotides.

The general pattern of affinity for the toxin correlates well with the inhibition of toxin attachment and action on cells, although the concentrations required for 50% inhibition of toxin binding obtained by Middlebrook and coworkers (3, 4) were generally 5- to 10-fold greater than the values of K_d we measured by flow dialysis. This difference is probably to be expected for at least two reasons. First, the measurement of toxin attachment to the cell surface involved incubation of cells with labeled toxin at 4°C for 12 hr, and the conditions at the end of this period probably did not represent an equilibrium state. Second, hydrolysis of nucleotides by free or cell-bound nucleotidases almost certainly occurred during the incubations. The fact that UTP and CTP were weaker inhibitors of toxin attachment than the other nucleoside triphosphates tested may have been due to base-specific nucleotidases or other complexities inherent in the cell-binding assay.

The stoichiometry of ATP binding revealed in the flow dialysis experiments implied that only a fraction of the molecules in any given toxin preparation could bind ATP in a rapidly reversible manner. To explore the question of heterogeneity, we chromatographed toxin on an ATP-affinity column. As shown in Fig. 2, approximately two-thirds of the toxin was unretarded and emerged in the void volume (fraction I). The remainder bound to the column and could be eluted with $0.5\ \text{M}$ NaCl (fraction II). Upon rechromatography under identical conditions, material from each fraction behaved essentially as it had on the initial column, with only traces of material (less than 10%) being shifted from one peak to the other. After concentrating and dialyzing both fractions, we measured the binding of ATP by flow dialysis (Fig. 3). Protein from fraction II bound ATP; in three experiments the values of K_d were 11.1, 12.5, and $13.6\ \mu\text{M}$, and values of n were 0.9, 0.91, and 1.1, respectively. No significant binding of ATP to fraction I could be detected. That the protein in both fractions was in fact

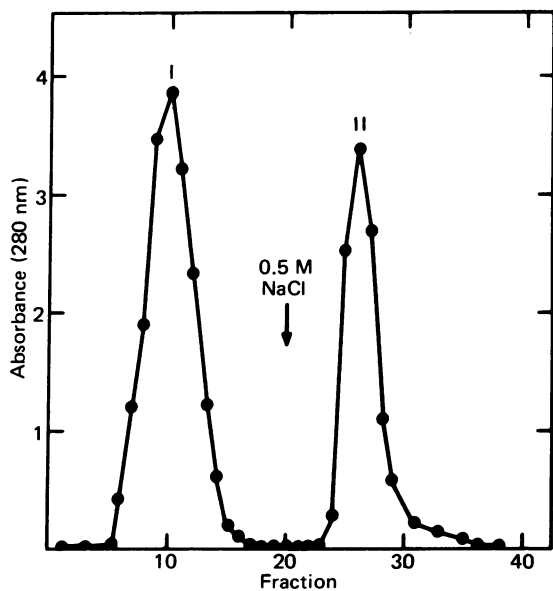


FIG. 2. Chromatography of diphtheria toxin on ATP-Sepharose. A 400- μ l sample of diphtheria toxin (9.6 mg/ml) was applied to a column of ATP-Sepharose (0.3 \times 3 cm), equilibrated in 50 mM Tris-HCl (pH 7.2). After the column had been washed in the same buffer and the absorbance at 280 nm had returned to base line, adsorbed material was eluted with 0.5 M NaCl in buffer. Fraction size was 0.2 ml.

diphtheria toxin was confirmed by the findings that: (i) both were equally toxic in our standard cell culture toxicity assays; (ii) tests in Ouchterlony plates indicated immunologic identity of the two fractions; and (iii) both gave the same band patterns on sodium dodecyl sulfate/polyacrylamide gels, before or after mild tryptic digestion and with or without reducing agent present.

A possible basis of the observed heterogeneity was revealed

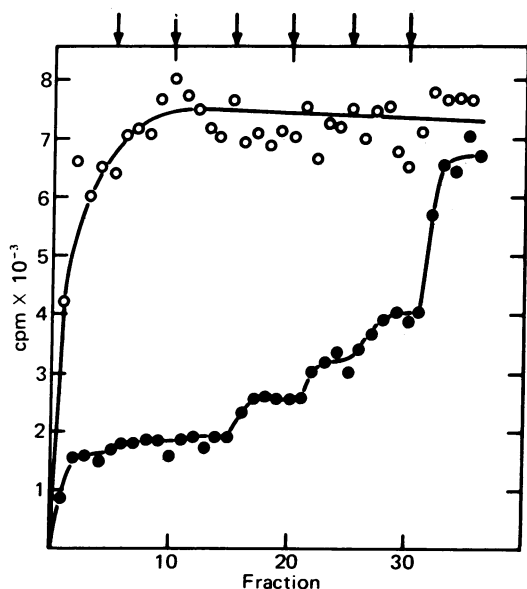


FIG. 3. Measurement by flow dialysis of ATP binding to fractions I and II from ATP-Sepharose. Fractions I and II, prepared as described in the legend of Fig. 2, were concentrated in a Speed Vac concentrator (Savant) and dialyzed against 50 mM Tris-HCl (pH 7.2). The conditions of flow dialysis were the same as for Fig. 1. Arrows indicate 1- μ l additions of 4.9 mM ATP to the upper chamber, containing 76 μ M fraction I (O) or 48 μ M fraction II (●).

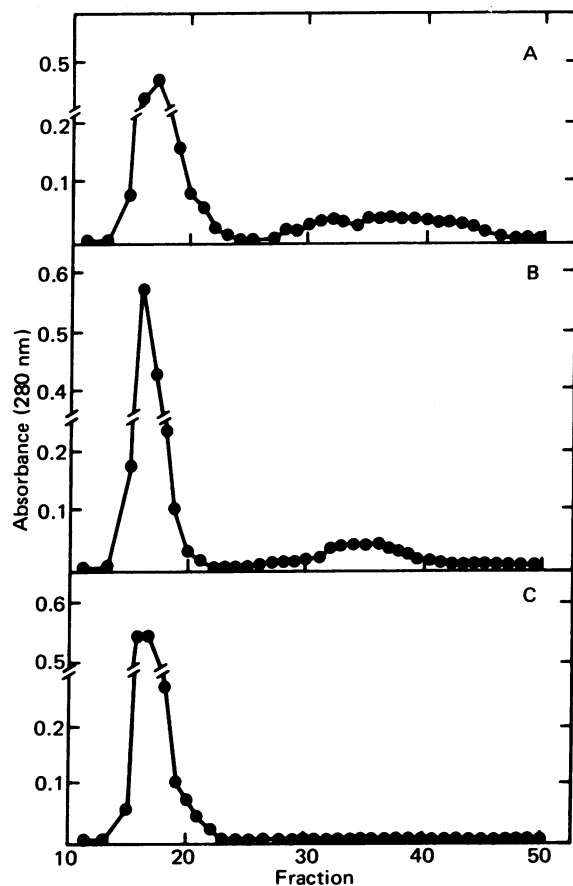


FIG. 4. Chromatography of diphtheria toxin and its subfractions on Sephadex G-50 in the presence of 6 M urea. A column of Sephadex G-50 (0.9 \times 11.5 cm) was equilibrated with 50 mM Tris-HCl, pH 7.2/6 M urea. Samples of toxin (A) or of fraction I (B) or II (C), prepared as described for Fig. 2 (100 μ g, 3 mg/ml), were equilibrated with 6 M urea, applied to the column, and eluted with urea-containing buffer at a flow rate of 10 ml/hr. Fractions of 200 μ l were collected, and the absorbance at 280 nm was measured.

in experiments in which toxin was incubated with 6 M urea and chromatographed over Sephadex G-50 in the presence of urea (Fig. 4). Such treatment caused release of a low molecular weight substance with an ultraviolet absorption profile resembling that of adenosine or uridine nucleotides ($\lambda_{max} = 260$ nm). When each of the two fractions from the ATP-Sepharose column was treated with urea and chromatographed, it was found that fraction I contained the 260 nm absorbing compound whereas fraction II did not. The ultraviolet absorption profiles of the two fractions and of unfractionated toxin were consistent with these results. Fraction II showed an absorption maximum of approximately 278 nm, whereas the maximum of fraction I was shifted to shorter wavelengths by 4–8 nm and its 260/280 ratio was substantially greater than that of fraction II. The absorption maximum of unfractionated toxin was intermediate between those of fractions I and II. The absorption profiles of the protein moieties of fractions I and II were identical after gel filtration in the presence of urea.

DISCUSSION

The affinity of ATP and related compounds for diphtheria toxin was found to correlate well with their inhibition of toxin action on cells. This inhibition, in turn, has been correlated with blocking of toxin attachment to cells (3–5) and appears unrelated to the toxin's intracellular action. Based on these results,

we propose that the inhibition of toxin attachment results from the binding of ATP or related compounds to the toxin molecule and not to the toxin receptor on the cell surface. Sperti and coworkers (12) reported binding of GTP to diphtheria toxin ($K_d = 5.9 \mu\text{M}$) in 1971, before it was known that nucleotides inhibit toxin attachment to cells.

The affinity of nucleotides for toxin was highly dependent on their phosphate content and largely independent of the nature of the purine or pyrimidine base, although the nucleoside moiety did contribute to the binding. The strong dependence of affinity on phosphate content, together with the competitive inhibition of ATP binding by polyphosphates, suggest that the site responsible for this binding may be strongly cationic. We propose that this site be designated the P site, based on its affinity for phosphates. The facts (see Table 1) that inositol hexaphosphate binds with high affinity and that trimetaphosphate, a cyclic form, binds with the same affinity as tripolyphosphate, a linear form, indicate that the site does not have rigid specificity with respect to the shape of the ligand.

Our results suggest that toxin receptors on the cell surface may contain a nucleotide, phosphate residues, or other negatively charged groups. Proia *et al.* (13) have isolated a glycoprotein fraction from guinea pig lymph-node cells that may correspond to the toxin receptor, but no studies of phosphate content were performed. The P site may be responsible for the binding of diphtheria toxin to phospholipid-containing vesicles, which has been reported recently (14).

The properties of the P site contrast strongly with those of the NAD^+ -binding site on free fragment A. Affinity of the NAD^+ -binding site for adenine is high and decreases as ribose and phosphate residues are added to the latter (10). However, as shown in Table 1, precisely the opposite is true for the P site. Although this suggests that the two sites may be separate and distinct, the possibility must be considered that there is partial overlap. Within the whole toxin, the NAD^+ -binding site on the fragment A moiety may be distorted such that its properties are significantly altered. Because whole toxin lacks ADP-ribosyltransferase activity, but exhibits some NAD^+ glycohydrolase activity (P. Bernard and R. J. Collier, unpublished data), the NAD^+ -binding site may be exposed, at least in a fraction of the molecules.

ATP does not bind to fragment A and ATP binding blocks attachment of toxin to cells; thus the P site may be localized to the toxin's B domain, which has been shown to function in attachment to receptors (15, 16). Furthermore, the fact that CRM45, which lacks the carboxyl-terminal M_r 17,000 region of the toxin, showed no detectable binding of ATP suggests that this latter region may contain (or contribute to) the P site. Analysis of ATP binding to fragment B by flow dialysis was not possible due to instability of the fragment at the concentrations necessary for measurement.

We were not entirely surprised to find that the number of binding sites for ATP was nonintegral and varied among preparations of toxin, inasmuch as similar results had been obtained for the binding of NAD^+ to whole toxin (n for NAD^+ ranged from less than 0.1 to as high as 0.7 among various toxin preparations; P. Bernard and R. J. Collier, unpublished data). Our results with both ligands implied molecular heterogeneity within individual toxin preparations. We were able to substantiate this heterogeneity by chromatography of toxin on an ATP affinity column and, furthermore, we demonstrated that a low molecular weight, 260-nm-absorbing substance was present on a fraction of the toxin that did not bind to the affinity resin. Release of a nucleotide or nucleotide-like compound by treatment of toxin with urea has also been observed in another laboratory (D. M. Neville, Jr., personal communication). This

substance was not removed by gel chromatography of the native toxin; it is firmly bound (perhaps to the P site). The structural basis of the toxin's heterogeneity may reside in the toxin *per se*; that is, there may be two forms of the toxin, one of which binds nucleotides very tightly. Alternatively, the endogenous nucleotide may form the basis of the strength of the toxin-nucleotide association. In the former case, the difference between the toxin forms must not depend on the degree of nicking; mild trypsin treatment of a preparation of largely intact molecules did not alter its ATP-binding properties.

Gill and Steinhaus (17) have reported the ADP-ribosylation of diphtheria toxin by itself, and it is conceivable that the endogenous nucleotide we report here may be related to this phenomenon. The self-ADP-ribosylation may occur in culture fluids or during toxin purification and may be mediated by NAD^+ released by cell leakage or lysis. Although the incorporated ADP-ribose is initially attached via covalent linkage, it was reported that the nucleotide was slowly released and that up to 40% of the protein-bound nucleotide became acid soluble during electrophoresis (17). The presence of ADP-ribose or other nucleotides bound to the toxin would be expected to alter several of its properties and may contribute to the reported isoelectric heterogeneity of purified toxin (18) as well as variations in other physical and biological properties among preparations (19, 20). Variability in the amount of endogenous nucleotide would affect the absorbance at 280 nm and, as a practical consequence, make this variable an unreliable means of estimating toxin concentration (19).

An unexpected finding was that ATP inhibited the action of both fractions of toxin, although fraction I did not bind ATP in a rapidly reversible manner. It may well be that the endogenous nucleotide on fraction I does, in fact, dissociate at a relatively low rate and can be replaced by ATP. Slow dissociation of the endogenous nucleotide may also explain the fact that fraction I was as toxic as fraction II in our routine cell-culture assay. The rate of dissociation, although too slow to permit detectable binding by flow dialysis, may nevertheless be sufficiently high to permit almost full toxicity to be manifested over the 24-hr duration of the assay. This would be consistent with binding of toxin to the cell surface receptor with a high affinity constant (values in the range of about 10^{-7} – 10^{-9} M have been reported; refs. 4, 5, and 21) or with its binding becoming rapidly irreversible (for example, by internalization or insertion into the plasma membrane). Also, the dissociated nucleotide might be hydrolyzed rapidly by cell-bound or free nucleotidases. It may be that differences in toxicity of the two fractions will be detectable in assays involving shorter incubation periods.

A low rate of ATP binding to fraction I of the toxin may also explain the difference between the K_d of ATP measured with unfractionated toxin ($18 \mu\text{M}$) and that obtained with fraction II ($\approx 12 \mu\text{M}$). Precise measurements by various methods will be necessary to obtain more accurate values of K_d and to define values for various association and dissociation rate constants.

When this work was partly completed, we learned from Leon Eidels that he and his coworkers, Richard L. Proia and David A. Hart, had obtained indirect evidence for binding of ATP to diphtheria toxin (22).

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