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Diphtheria Toxin Can Simultaneously Bind to Its Receptor and Adenylyl-(3′,5′)-uridine 3′-Monophosphate†

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ABSTRACT: Diphtheria toxin that was bound to receptors on BS-C-1 cells was able to bind approximately 1 molar equiv of adenylyl-(3′,5′)-uridine 3′-monophosphate (ApUp). In contrast, receptor-bound CRM197, a mutant form of toxin with greatly diminished affinity for dinucleotides, did not bind ApUp. Affinity of the dinucleotide for receptor-bound toxin differed from that for free toxin by less than an order of magnitude. These results indicate that the receptor site and the ApUp site on the toxin do not significantly overlap.

There is evidence suggesting that the receptor site and the dinucleotide site on diphtheria toxin (DT)[1]—which perform functionally and temporally distinct events in the intoxication process and lie on opposite ends of the primary structure—may overlap in the native toxin. Compounds containing multiple phosphate residues, such as nucleoside triphosphates or inositol hexaphosphate, inhibit both binding of the toxin to cells (Middlebrook & Dorland, 1979) and binding of NAD or the endogenous dinucleotide, adenylyl-(3′,5′)-uridine 3′-monophosphate (ApUp), to the toxin (Lory et al., 1980; Collins & Collier, 1984). A variety of evidence supports a model in which the dinucleotide binding site on the A moiety is in close proximity to a cationic, phosphate-binding region (P-site) on the B moiety (Lory et al., 1980; Proia et al., 1981). It has therefore been suggested that the P-site might actually correspond to the receptor site. If this were true, one would expect that association of the toxin with its receptor would prevent binding of dinucleotides.

To test this possibility we took advantage of the high affinity of ApUp for the dinucleotide site on DT. ApUp (whose source and function are uncertain) is tightly bound to a fraction of the toxin molecules in many preparations (Barbieri et al., 1981). Its binding is competitive with respect to NAD and various phosphorylated compounds (Collins & Collier, 1984). At 5 °C in 50 mM Tris-HCl buffer, pH 7.1, the K_d of ApUp for the toxin in solution is 9 pM and the half-life of the toxin-ApUp complex is about 60 min (Collins et al., 1984). Thus if receptor-bound toxin were able to bind ApUp with a similar affinity, the complex should be sufficiently stable to survive brief washing and allow accurate quantification. We therefore performed experiments to determine if toxin bound to receptors

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[1] Abbreviations: DT, diphtheria toxin; ApUp, adenylyl-(3′,5′)-uridine 3′-monophosphate; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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on BS-C-1 cells could form a complex with radiolabeled ApUp.

Experimental Procedures

Materials. Diphtheria toxin was purchased from Connaught Laboratories and purified as previously described (Collier & Kandel, 1971). Monomeric diphtheria toxin was chromatographed on Amicon Green A to separate the nucleotide-bound and -free forms of the toxin (Carroll et al., 1986). Nicked monomeric nucleotide-free diphtheria toxin was used throughout this study. CRM197 was prepared from *Corynebacterium diphtheriae* C7 (β crm197) and purified as described (Uchida et al., 1973).

Radiolabeled nucleotides and iodine were from Amersham Corp., and lactoperoxidase and egg albumin were from Calbiochem. ApUp and [^{32}P]ApUp were prepared as previously described (Collins et al., 1984). BS-C-1 cells (African Green Monkey kidney cells; ATCC CCL 26) were purchased from the American Type Culture Collection.

Radioiodination of Proteins. Proteins were labeled with ^{125}I by using lactoperoxidase (Roth, 1975). The reaction mixture (100 μL) contained 38 μg of target protein, 0.1 M sodium acetate, pH 5.6, 2.0 mCi of ^{125}I, 0.13 μM lactoperoxidase, and 0.000 05% H_2O_2. H_2O_2 was added last to start the reaction. After 30 s at room temperature, 100 μL of 1.0% NaI and 0.02% NaN_3 was added and the reaction mixture was chromatographed over Sephadex G-50 equilibriated in 50 mM Tris-HCl, pH 7.4, containing 0.1% bovine serum albumin. Protein fractions were pooled and stored at 5 °C. Specific activities ranged from 1×10^7 to 5×10^7 cpm per microgram of protein, as determined by liquid scintillation. Radioiodinated DT bound reversibly to Amicon Green A, which indicated that the dinucleotide binding site remained functional. The specific cytotoxicity of radioiodinated DT was equal to that of unmodified toxin.

Binding of DT or CRM197 to Cells. Twenty-four well microtiter plates were seeded with 5×10^4 BS-C-1 cells per well and incubated for 24–48 h. Confluent cell lawns were rinsed 1 time with cold Dulbecco's phosphate-buffered saline, pH 7.4 (PBS), and incubated at 5 °C in 0.5 mL of DMEM containing 25 mM HEPES, pH 7.4, and 0.05% egg albumin (binding buffer). Radiolabeled protein was added, and after incubation for the times indicated, the cells were washed 4 times with PBS. Next, radiolabeled ApUp was added and incubations were continued. At the indicated times, cells were washed 4 times with PBS and dissolved in 0.1 N NaOH, and solubilized cell material was assayed for radioactivity. Results shown are the average of duplicate measurements in a single experiment. The results were confirmed in at least two independent experiments.

DT binding to BS-C-1 cells was specific and saturable. Specific association was determined as the difference between the total radiolabeled DT bound to cells in the absence and presence of 100-fold excess nonradiolabeled DT. Specific association was about 90% of the total with DT and 70% with CRM197 under the conditions chosen. At 5 °C, the binding of 1.0 nM DT to BS-C-1 cells was linear for about 4 h, reached 80% of maximal by 8 h, and reached a plateau by 16 h. Scatchard analysis was consistent with the presence of one class of DT receptors, with an apparent equilibrium dissociation constant of 0.7 nM.

Results

DT Can Simultaneously Bind Receptors and ApUp. BS-C-1 cells were incubated at 5 °C with or without ^{125}I-labeled DT or CRM197 and washed. They were then incubated with [^{32}P]ApUp, washed, and assayed for the two radiolabels.

Table I: ApUp Binding to BS-C-1 Cells Preincubated with DT or CRM197[a]

	cell-associated radiolabel[b]		
protein	^{125}I-protein [cpm (fmol)]	[^{32}P]ApUp [cpm (fmol)]	ApUp/protein (ratio)
DT	6892 (2.3)	10300 (2.8)	1.2
CRM197	2607 (1.8)	441 (<0.1)	<0.1
none	12 (<0.1)	418 (<0.1)	

[a] BS-C-1 cells were incubated for 16 h at 5 °C alone, with 1.0 nM ^{125}I-DT (specific activity 3.0×10^6 cpm/pmol) or with 1.0 nM ^{125}I-CRM197 (specific activity 1.1×10^6 cpm/pmol). Cells were washed 4 times with cold PBS and covered with 0.25 mL of binding buffer containing 1 nM [^{32}P]ApUp (specific activity 5×10^6 cpm/pmol). After 15 min, the cells were washed 4 times with cold PBS and dissolved in 0.4 mL of 0.1 N NaOH, and radioactivity was measured by liquid scintillation. [b] Nonspecific cell-associated radiolabel has been subtracted as described under Experimental Procedures.

Cells that had been exposed to DT bound approximately stoichiometric amounts of ApUp (Table I). Cells not treated with DT or those incubated with CRM197 instead of DT bound less than 5% as much ApUp. CRM197 is a nontoxic mutant of DT that binds to sensitive cells but has a greatly reduced affinity for dinucleotides (Collins & Collier, 1984). Its K_d for ApUp in solution at 25 °C is 1 μM, which represents an affinity 5000-fold lower than that observed with native toxin.

About 90% of the ^{125}I-DT bound to BS-C-1 cells was displaced in the presence of 100-fold excess of unlabeled toxin, and we estimated that the cells contained about 32 000 receptors per cell.

Kinetics of Association and Dissociation of ApUp with Receptor-Bound Toxin. The rate of release of [^{32}P]ApUp from receptor-bound DT was measured at 5 °C in the presence of 500-fold excess nonlabeled ApUp. The release was first order, with a half-life of 19 min, corresponding to a dissociation rate constant of 6.0×10^{-4} s^{-1} (Figure 1). Only slight dissociation of the labeled toxin from cells was observed over the period of the experiment.

The rate of association of ApUp with receptor-bound DT was also determined. BS-C-1 cells were preincubated with ^{125}I-DT, washed, incubated for various periods with [^{32}P]-ApUp, and washed again. ApUp binding reached saturation by 30 min, with approximately 1 mol of ApUp associated per mole of receptor-bound DT (Figure 2). We calculated the association rate constant to be 2.5×10^6 s^{-1} M^{-1}. From the association and dissociation rate constants we calculated a value of 250 pM for the K_d of ApUp for receptor-bound DT under the conditions employed.

The dissociation rate constant was about 3-fold greater than that measured in solution, while the association rate constant was about 10-fold lower (Collins et al., 1984). The combined differences represent approximately 30-fold lower affinity of ApUp for DT when the toxin is bound to BS-C-1 cells. The toxin's affinity for ApUp in solution was shown to diminish by a factor of 5 when the ionic strength was increased by 100 mM (Collins et al., 1984). Thus the higher ionic strength of the cell binding medium (ca. 160 mM, compared with ca. 50 mM for the measurements in solution) can account for about half the differences in affinity observed. Conformational alterations induced by interaction of toxin with its receptor may also affect the affinity of ApUp binding. We have no data regarding possible effects of specific ions.

Acidification of the Cell-Bound ApUp–DT Complex. Acidification of receptor-bound DT is believed to play an important role in the process of intoxication (Sandvig & Olnes,

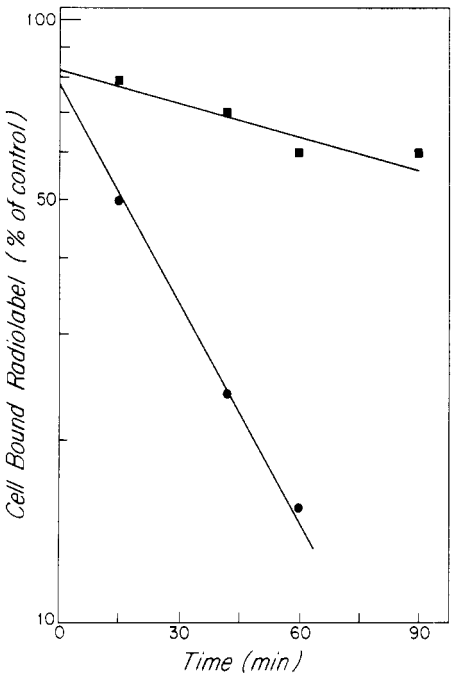

FIGURE 1: Kinetics of dissociation of ApUp from receptor-bound DT. Duplicate samples of BS-C-1 cells were washed with PBS and incubated at 5 °C with 0.5 mL of binding buffer prechilled to that temperature. ^{125}I-DT (1 nM; specific activity 3.0×10^6 cpm/pmol) was added, and after 16 h at 5 °C, the cell lawns were washed 4 times with PBS. Next cells were incubated with 0.25 mL of binding buffer containing 1 nM [^{32}P]ApUp (specific activity 4×10^6 cpm/pmol) for 30 min, at which time cell lawns were washed 3 times with cold PBS. The cells were then incubated in binding buffer (5 °C) containing 1 μg/mL DT and 1 μM ApUp, and at the indicated times cell-bound radioactivity [DT (■); and ApUp (●)] was determined as described in Table I.

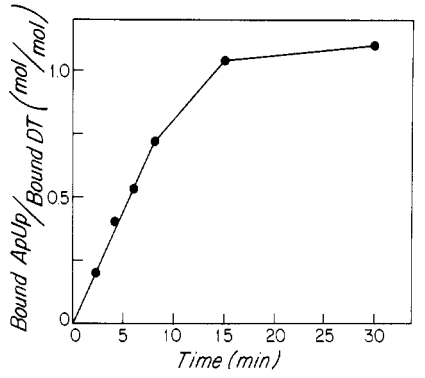

FIGURE 2: Kinetics of ApUp binding to BS-C-1 cells preincubated with diphtheria toxin. BS-C-1 cells were washed with PBS and incubated in 0.5 mL of binding buffer prechilled to 5 °C. ^{125}I-DT (1 nM; specific activity 2.5×10^6 cpm/pmol) was added to the binding buffer alone or with 100-fold excess nonradiolabeled DT, and the incubation was continued for 16 h when the cells were washed 4 times with PBS. Next [^{32}P]ApUp (0.9 nM; specific activity 1.5×10^6 cpm/pmol) in 0.25 mL of binding buffer was added and the incubation continued for the indicated time, when cells were washed 3 times with PBS. Cell-bound radioactivity was determined as described in Table I. (●) represents the molar ratio of cell-bound ApUp and DT.

1980; Draper & Simon, 1980;, Donovan et al., 1981; Kagan, et al., 1981). The receptor-bound toxin is endocytosed within coated vesicles and introduced into endosomes. As the lumenal pH declines, the toxin inserts into the vesicular membrane and transfers the A chain to the cytosolic surface. There is evidence that this insertion and transfer of A are mimicked when receptor-bound toxin at the cell surface is briefly exposed to mildly acidic conditions (Sandvig & Olnes, 1980; Draper & Simon, 1980).

Table II: Release of ApUp from Receptor-Bound DT by Acidification[a]

	cell-associated radiolabel[b]		
treatment (pH)	^{125}I-DT [cpm (fmol)]	[^{32}P]ApUp [cpm (fmol)]	ApUp/DT (ratio)
A, none	3405 (1.2)	5859 (1.2)	1.0
B (4.5)	2747 (0.9)	134 (<0.1)	0.0
C (7.4)	2909 (1.0)	3174 (0.6)	0.6

[a] All operations were performed at 5 °C. BS-C-1 cells were incubated for 16 h with 1.0 nM ^{125}I-DT (specific activity 2.9×10^6 cmp/pmol), washed 4 times with cold PBS, and incubated 15 min with 0.25 mL of binding buffer containing 1.5 nM [^{32}P]ApUp (specific activity 4.9×10^6 cpm/pmol). Next cells were washed 4 times with PBS and assayed immediately (A) or incubated at either pH 4.5 for 5 min (B) or pH 7.4 for 5 min (C) prior to measurement of cell-bound radiolabel, which was determined as described in Table I. [b] Nonspecific cell-associated radiolabel has been subtracted as described under Experimental Procedures.

BS-C-1 cells containing prebound, differentially labeled DT and ApUp were treated for 5 min at pH 4.5, washed, and assayed for the two labels. As shown in Table II, acidification caused virtually complete release of the ApUp but release of only 10% of the DT, relative to controls maintained at pH 7.4. This finding correlates with results showing that exposure of toxin in solution to a similar pH (pH 4.7) decreased its affinity for ApUp by >4 orders of magnitude (Collins, 1984).

Discussion

The results presented demonstrate that DT can simultaneously bind to ApUp and to receptors on BS-C-1 cells. CRM197 provided an excellent control, since it was capable of binding to receptors but was unable to bind the dinucleotide. These results constitute direct evidence that the ApUp and receptor binding sites on the toxin do not overlap. Other results are consistent with this notion: (i) CRM197 binds ApUp with greatly diminished affinity compared with DT, while its receptor-binding properties remain closely similar to those of the toxin (Collins & Collier, 1984; Mekada & Uchida, 1985); (ii) dimeric DT has a high-affinity ApUp site but does not bind to receptors (Carroll et al., 1986). Other investigators have reported data consistent with a functional separation of the P-site and the receptor binding site of the toxin (Eidels et al., 1982; Boquet & Duflot, 1981). The results presented here also suggest that inhibition of receptor binding by phosphorylated compounds may occur via interaction of such compounds with a different site on the toxin, possibly the receptor binding site per se.

After correction for differences in ionic conditions of the measurements, the affinity of ApUp for cell-bound toxin was within an order of magnitude of that for free toxin. The toxin's interaction with its receptor may induce conformational changes that slightly diminish affinity for ApUp and account for the differences seen. The results presented imply that radiolabeled ApUp is potentially useful as a reagent for quantifying receptor-bound toxin at the cell surface and for differentiating between the native molecule and acidic, conformationally altered forms.

Treatment at pH 4.5 induced quantitative release of ApUp bound to toxin on the cell surface, while releasing only about 10% of toxin. This result was consistent with the finding of a marked decrease in affinity of toxin for ApUp as the pH is lowered below 5. Various results indicate that acidic pH in this range causes conformational changes that induce insertion of membrane-bound toxin, but in solution result in aggregation and partial denaturation of toxin (Collins, 1984;

Blewitt et al., 1985). The results also imply that ApUp bound to toxin within an endosome would probably not prevent pH-induced membrane insertion and subsequent steps in the intoxication process. Under our experimental conditions, we did not observe a rapid release of DT from cell surface receptors, which is consistent with the insertional model.

Eidels and co-workers reported that under certain experimental conditions the nucleotide-bound form of diphtheria toxin was several hundredfold less toxic than the nucleotide-free form of the toxin (Proia et al., 1981). They attributed this decrease in cytotoxic potential to the inability of the nucleotide-bound form of the toxin to interact with cell surface receptors. Our observation that the ApUp binding site on receptor-bound toxin is accessible and not greatly perturbed conflicts with this conclusion. In a separate communication we report results on the effect of dinucleotides, including ApUp, on the cytotoxic action of DT.

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Phenylalanine Hydroxylase from *Chromobacterium violaceum* Is a Copper-Containing Monooxygenase. Kinetics of the Reductive Activation of the Enzyme†

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ABSTRACT: Pterin-dependent phenylalanine hydroxylase from *Chromobacterium violaceum* contains a stoichiometric amount of copper (Cu^{2+}, 1 mol/mol of enzyme). Electron paramagnetic resonance spectroscopy of the enzyme indicates that it is a type II copper-containing protein. The oxidized enzyme must be reduced by a single electron to be catalytically active. Dithiothreitol was found to be an effective reducing agent for the enzyme. Electron paramagnetic resonance data and kinetic results indicate the formation of an enzyme–thiol complex during the aerobic reduction of the enzyme by dithiothreitol. 6,7-Dimethyltetrahydropterin also reductively activates the enzyme, but only in the presence of the substrate, and is kinetically less effective than dithiothreitol. The metal center is not reoxidized as a result of normal turnover. However, the data indicate an alternative pathway exists that results in slow reoxidation of the enzyme. The 4a-hydrate of 6-methyltetrahydropterin (4a-carbinolamine) is observed during turnover of the enzyme. This intermediate is also observed during the reaction catalyzed by the iron-containing mammalian enzyme, suggesting that the mechanism of oxygen activation is similar for both enzymes.

Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) catalyzes the stoichiometric formation of tyrosine from phenylalanine and molecular oxygen. The enzyme requires a tetrahydropterin cofactor, which is oxidized by two electrons during each turnover, for activity. The mammalian enzyme contains 1 mol equiv of essential non-heme iron (Fisher et al., 1972; Gottschall et al., 1982). For catalytic activity ferric phenylalanine hydroxylase (PAH)[1] must be reduced to

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