Binding of ATP by Pertussis Toxin and Isolated Toxin Subunits†

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ABSTRACT: The binding of ATP to pertussis toxin and its components, the A subunit and B oligomer, was investigated. Whereas, radiolabeled ATP bound to the B oligomer and pertussis toxin, no binding to the A subunit was observed. The binding of [^{3}H]ATP to pertussis toxin and the B oligomer was inhibited by nucleotides. The relative effectiveness of the nucleotides was shown to be ATP > GTP > CTP > TTP for pertussis toxin and ATP > GTP > TTP > CTP for the B oligomer. Phosphate ions inhibited the binding of [^{3}H]ATP to pertussis toxin in a competitive manner; however, the presence of phosphate ions was essential for binding of ATP to the B oligomer. The toxin substrate, NAD, did not affect the binding of [^{3}H]ATP to pertussis toxin, although the glycoprotein fetuin significantly decreased binding. These results suggest that the binding site for ATP is located on the B oligomer and is distinct from the enzymatically active site but may be located near the eukaryotic receptor binding site.

Pertussis toxin, an exotoxin, produced by *Bordetella pertussis*, is believed to be involved in the pathogenesis of the organism (Manclark & Cowell, 1984). This toxin acts by catalyzing the ADP-ribosylation of a family of GTP-binding regulatory proteins (G proteins) found in eukaryotic cells (Katada & Ui, 1982a,b; Gilman, 1984; Bokoch et al., 1984; Manning & Gilman, 1983; Sternweis & Robishaw, 1984). When ADP-ribosylated, the G proteins loose their ability to mediate signal transduction within the eukaryotic cell. Thus the cell no longer responds to a variety of hormones and neurotransmitters (Hazeki & Ui, 1981; Burns et al., 1983; Okajima & Ui, 1984; Nakamura & Ui, 1985; Bokoch & Gilman, 1984; Kikuchi et al., 1986; Asano et al., 1985).

Pertussis toxin is a typical bacterial toxin in that it has an A–B structure that comprises an enzymatically active A subunit and a B oligomer, which is responsible for binding of the toxin to the eukaryotic cell (Tamura et al., 1982; Gill, 1978; Moss & Vaughan, 1979). The B oligomer is composed of five subunits ranging in molecular weight from 11 000 to 22 000 (Tamura et al., 1982; Locht & Keith, 1986; Nicosia et al., 1986). The A subunit is a single polypeptide chain that contains the ADP-ribosyltransferase activity of the toxin (Katada et al., 1983). In the absence of the G protein substrate, this subunit catalyzes the hydrolysis of NAD to ADP-ribose and nicotinamide (Katada et al., 1983; Moss et al., 1983). This is an abortive reaction in which H_2O rather than a protein substrate serves as an acceptor for the ADP-ribosyl moiety of NAD.

The toxin is released from the bacterium in an inactive form. ATP is required for manifestation of enzymatic activity (Katada & Ui, 1982b; Lim et al., 1985). This nucleotide appears to activate the toxin by promoting the dissociation of the A subunit from the B oligomer thereby releasing the constraints imposed by the B oligomer on the A subunit (Burns & Manclark, 1986). Little is known concerning the location of the ATP binding site on the pertussis toxin molecule. Elucidation of the location of this site would aid in the understanding of the regulation of pertussis toxin activity. In this report, we demonstrate direct binding of ATP to the B oligomer and have examined this binding in detail.

Materials and Methods

Materials. Pertussis toxin was purchased from the Michigan Department of Public Health. Nitrocellulose filters (0.45 μm) were from Schleicher and Schuell, Keene, NH, CM Sepharose was purchased from Pharmacia, Piscataway, NJ, CHAPS[1] was from Calbiochem-Behring, La Jolla, CA, and [^{3}H]ATP (51.8 Ci/mmol) was purchased from NEN-Du Pont, Wilmington, DE. Dowex 1 × 8 resin was from Bio-Rad, Richmond, CA. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Goose erythrocytes were obtained from Truslow Farms, Chestertown, MD.

Protein Determination. Protein values were determined according to the method of Bradford, using ovalbumin as a standard (Bradford, 1976).

Isolation of Toxin Subunits. B oligomer was separated from the A subunit according to the method previously described (Burns et al., 1987).

Binding Assay. A filter binding assay was used to measure the amount of radiolabeled ATP bound to pertussis toxin or its subunits. Pertussis toxin (1.5 μg), B oligomer (1.0 μg), or A subunit (1.0 μg) in a solution containing [^{3}H]ATP (approximately 150 000 counts per assay), 25 μg ovalbumin, other compounds as indicated, and either 50 mM potassium phosphate, pH 7.5, or 50 mM Tris-HCl buffer, pH 7.5 (total assay volume = 100 μL) was incubated at 25 °C for 10 min. The reaction mixtures were then placed on ice, filtered through nitrocellulose, and washed twice with ice cold buffer. The amount of [^{3}H]ATP bound to the filters was determined by using a liquid scintillation counter. Unless otherwise indicated, the assay mixture contained 50 mM Tris-HCl, pH 7.5, when ATP binding to pertussis toxin was examined and it contained 50 mM potassium phosphate, pH 7.5, when ATP binding to B oligomer was examined. Data are means of values from triplicate determinations. Control experiments demonstrate that [^{3}H]ATP does not dissociate from either pertussis toxin

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethane sulfonic acid; ApUp, adenylyl-(3′,5′)-uridine 3′-monophosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; PT, pertussis toxin.

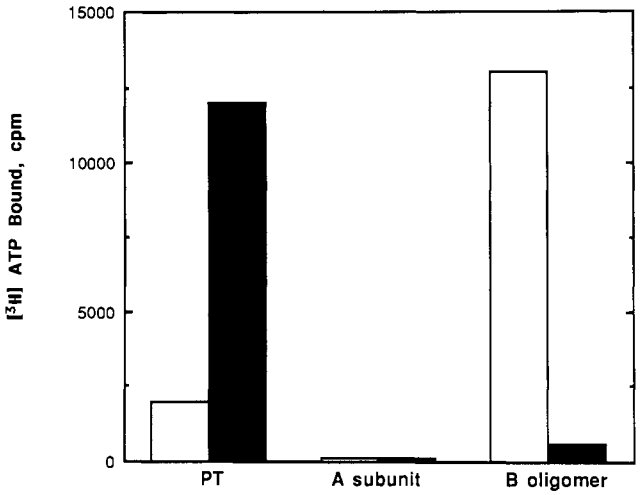

FIGURE 1: Binding of [^{3}H]ATP to pertussis toxin, A subunit, and B oligomer. Binding of 0.15 μM [^{3}H]ATP to the indicated protein was measured as described under Materials and Methods. Assay mixtures contained either 50 mM potassium phosphate buffer, pH 7.0 (open bars), or 50 mM Tris-HCl buffer, pH 7.0 (solid bars).

or B oligomer during the washing procedures. Furthermore, incubation of the proteins with [^{3}H]ATP for periods greater than 10 min does not increase the extent of binding (data not shown).

Hemagglutination Reaction. The ability of pertussis toxin to agglutinate goose red blood cells was evaluated according to the method previously described (Burns et al., 1987) except that TBS, pH 7.0, was substituted for PBS, pH 7.0.

Determination of Purity of ATP. The radiolabeled ATP employed in the binding assays was measured for the presence of contaminating ADP, AMP, and adenosine by a modification of the method of Thompson (Thompson et al., 1969). Dowex 1 × 8 (200–400 mesh) was washed successively three times with 2 N hydrochloric acid (10 mL), 2 N sodium hydroxide (10 mL), and water (10 mL). [^{3}H]ATP (3 700 000 cpm) was added to a 1.0 × 1.0 cm column of the Dowex and contaminating materials were eluted as follows: adenosine with deionized water (24 mL), AMP with 0.01 N hydrochloric acid (24 mL), ADP with 0.02 M sodium chloride in 0.02 N hydrochloric acid (24 mL), and ATP with 0.2 M sodium chloride in 0.02 N hydrochloric acid (24 mL). The amount of radioactivity eluting in each sample was measured by using a liquid scintillation counter. Purity of the [^{3}H]ATP preparations used in this study was determined to be approximately 60%.

The purity of unlabeled ATP was also measured by this method except that contaminants were monitored by absorbance at 257 nm. No adenosine, AMP, or ADP was found to contaminate the ATP. Recovery of ATP from the column was approximately 93%.

Results

The binding of [^{3}H]ATP to pertussis toxin and its isolated A and B components was examined. As shown in Figure 1, direct binding of ATP to both the holotoxin and the B oligomer was detected when the assay was conducted with a potassium phosphate buffer (pH 7.5). Under these conditions, the B oligomer bound the radiolabeled nucleotide to a greater extent than the holotoxin molecule. No binding of [^{3}H]ATP to the A subunit was observed. Similar results were obtained when a sodium phosphate buffer (pH 7.5) was employed (data not shown). Although ATP has been reported to dissociate pertussis toxin into its A and B components (Burns & Manclark, 1986), it does so only in the presence of the detergent CHAPS. Therefore, under the conditions of this binding assay, no dissociation of pertussis toxin should occur. When the phosphate buffer was replaced by Tris-HCl buffer (pH 7.5), the ability of the proteins to bind ATP was altered dramatically. The extent of binding of the nucleotide to the holotoxin increased whereas binding to the B oligomer was almost abolished. Again, the A subunit did not bind ATP. When the assays were conducted with HEPES or TES buffer, pH 7.5, the results were similar to those obtained when Tris-HCl was employed as the buffer (data not shown). These results suggest that phosphate may affect the ability of both the holotoxin and the B oligomer to bind ATP, although in opposite manners. Therefore, we further examined the effect of phosphate on the binding of ATP to these proteins.

Inorganic phosphate inhibited the binding of ATP to the holotoxin molecule in a competitive manner (data not shown). Pyrophosphate and tripolyphosphate, which may resemble the phosphate moiety of ATP, also inhibited ATP binding and were more effective inhibitors than phosphate, sulfate, and arsenate (Table I). The effect of phosphate and pyrophosphate on ATP binding to the B oligomer was more complex (Figure 2). At low concentrations, both phosphate and pyrophosphate enhanced the ability of the B oligomer to bind

Table I: Inhibition of [^{3}H]ATP Binding to Pertussis Toxin and B Oligomer[a]

compound	IC_{50} PT	IC_{50} B oligomer
tripolyphosphate[b]	30 μM	ND[c]
pyrophosphate[b]	100 μM	ND
potassium phosphate	500 μM	ND
arsenate[b]	2 mM	ND
sulfate[b]	2 mM	ND
ATP	100 nM	500 nM
GTP	20 μM	100 μM
CTP	200 μM	1 mM
TTP	300 μM	300 μM

[a] The binding of [^{3}H]ATP to pertussis toxin and B oligomer was measured in the presence of various concentrations of the indicated compounds. Each solution of inhibitor was adjusted to a pH of 7.5 before addition of toxin or B oligomer. PT or B oligomer was incubated with the inhibitors for 10 min prior to the addition of the ATP mixture. The extent of binding was determined as described under Materials and Methods. Binding in the absence of inhibitor was measured. The amount of inhibitor needed to decrease this binding by 50% is reported as the IC_{50}. [b] Sodium salt. [c] ND = not determined.

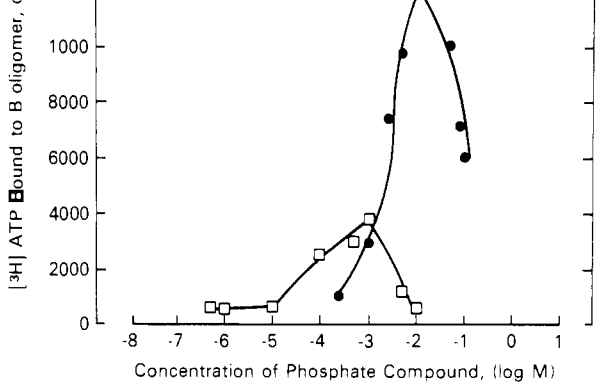

FIGURE 2: Effect of phosphate on the binding of [^{3}H]ATP to B oligomer. The binding of [^{3}H]ATP to B oligomer in the presence of 50 mM Tris-HCl, pH 7.5, and the indicated concentrations of sodium pyrophosphate, pH 7.0 (□), or potassium phosphate, pH 7.0 (●), was measured. B oligomer was incubated with the compounds under investigation for 10 min prior to the addition of the ATP mixture. The extent of binding was determined as described under Materials and Methods.

Table II: Effect of Nucleotides and Proteins on Binding of ATP to Pertussis Toxin

additions	% binding[a]	additions	% binding[a]
none	100 ± 7	NAD (50 μM)	103 ± 10
fetuin (100 μg/mL)	61 ± 2	ApUp (50 μM)	90 ± 6

[a] Values are expressed as percentage of binding in the presence of the indicated compound compared to binding in its absence and represent means of triplicate values ± SD.

ATP, whereas at higher concentrations, these compounds inhibited binding.

The binding of ATP to both pertussis toxin and the B oligomer was inhibited by nucleotides (Table I). The relative effectiveness of these compounds in inhibiting ATP binding to the holotoxin is similar to their effectiveness in inhibiting binding to B oligomer.

The parameters for binding of ATP to pertussis toxin and the B oligomer were examined. Dissociation constants calculated from Scatchard analysis of the data were 0.017 μM for pertussis toxin and 0.37 μM for the B oligomer. (These dissociation constants are not directly comparable since the assays were conducted with different buffers for the two species.) The average number of binding sites per molecule was 0.2 for pertussis toxin and 0.46 for B oligomer. Two different pertussis toxin preparations were studied and neither was found to have an integral number of binding sites.

Since the B oligomer also contains the binding site for the eukaryotic receptor, we examined the possibility that the ATP binding site and the receptor binding site might overlap. Fetuin, a glycoprotein that is believed to be a model for the eukaryotic receptor (Armstrong et al., 1988; Witvliet et al., 1989), decreased ATP binding to pertussis toxin by approximately 40% (Table II). The ability of ATP to bind to the eukaryotic receptor site of the toxin was assayed by examining the effect of ATP on the ability of pertussis toxin to bind and agglutinate red blood cells. ATP at concentrations as high as 3 mM had no effect on the hemagglutination induced by pertussis toxin (data not shown). In contrast, fetuin (625 μg/mL) and sialic acid (150 mM), which are believed to mimic the eukaryotic receptor and thus bind to and block the cellular binding site on the toxin (Armstrong et al., 1988; Witvliet et al., 1989), inhibited hemagglutination.

We also examined the effect of NAD, which is a substrate for pertussis toxin, on ATP binding to the toxin. NAD had no detectable affect on ATP binding (Table II). ApUp, which binds with high affinity to diphtheria toxin (Barbieri et al., 1981), was assayed for its ability to inhibit ATP binding to pertussis toxin. This compound had little effect on ATP binding to the toxin.

Discussion

The data presented in this report suggest that the ATP binding site of pertussis toxin is located on the B oligomer. The relative abilities of nucleotides to inhibit binding of ATP to pertussis toxin and the B oligomer correlate well with the reported potencies of these nucleotides in promoting dissociation of pertussis toxin subunits and thus activation of the A subunit (Burns & Manclark, 1986). Previously, ATP (in the presence of the detergent CHAPS) was shown to be effective in dissociating pertussis toxin subunits. GTP was less effective than ATP whereas CTP and TTP had little effect on the subunit structure of the toxin (Burns & Manclark, 1986).

Phosphate-containing compounds affected the binding of ATP to the holotoxin molecule and the B oligomer. Phosphate has previously been shown to inhibit ATP binding to the holotoxin molecule (Mattera et al., 1986). Phosphate binding to pertussis toxin exhibits competitive inhibition with respect to ATP binding, suggesting that phosphate ions bind to the ATP binding site on the pertussis toxin molecule. Binding of ATP to the B oligomer, in contrast, is enhanced by low concentrations of phosphate or pyrophosphate. High concentrations of these compounds, on the other hand, inhibit binding of ATP to the B oligomer. These results are consistent with the hypothesis that binding of phosphate-containing compounds to the B oligomer stabilizes a form of the molecule that binds ATP with high affinity. At subsaturating concentrations of phosphate or pyrophosphate, ATP would effectively compete with these compounds and binding of radiolabeled ATP would be observed. High concentrations of phosphate (>10 mM) and pyrophosphate (>1 mM) would saturate the nucleotide binding site(s) on the molecule, thus blocking ATP binding. While phosphate-containing compounds enhance binding of ATP to the B oligomer, a similar phenomenon is not observed with the holotoxin molecule. The molecular basis for the different effects of phosphate on ATP binding to holotoxin and B oligomer is not clear at this time. Perhaps the holotoxin molecule remains in a conformation that binds ATP with high affinity even in the absence of phosphate.

ATP binds with high affinity to the holotoxin. The number of binding sites on pertussis toxin for ATP was nonintegral and did not vary among the two toxin preparations tested. Only 0.2 mol of ATP bound to each mole of pertussis toxin. We do not know why a nonintegral number of sites was measured. Similar results were obtained when binding of ATP to another bacterial toxin, diphtheria toxin, was first examined (Lory & Collier, 1980). Early reports of the stoichiometry of ATP binding to diphtheria toxin indicated that only a fraction of the toxin molecules in a preparation bound ATP in a rapidly reversible manner. Subsequently, this heterogeneity was found to be due to the presence of an endogenous nucleotide, ApUp, which remained tightly bound to a fraction of the toxin molecules (Barbieri et al., 1981). We examined the ability of ApUp to compete with ATP binding to pertussis toxin. The concentration of ApUp needed for 50% inhibition of ATP binding was >500 μM. Thus this nucleotide does not bind tightly to the ATP binding site of pertussis toxin. Previously, ApUp was found to have no effect on the enzymatic activity of pertussis toxin (Kaslow et al., 1987). We do not know whether another tightly bound endogenous molecule that blocks the ATP binding site may account for the low stoichiometry of binding of ATP to pertussis toxin that we observe.

The eukaryotic receptor for pertussis toxin is believed to be the N-linked carbohydrate chains of certain cellular glycoproteins (Brennan et al., 1989; Witvliet et al., 1989). The structure of the carbohydrate moiety on the cellular receptor is similar to the N-linked carbohydrate chains on fetuin (Sekura et al., 1985; Witvliet et al., 1989). Fetuin has therefore been used as a model for the eukaryotic receptor (Sekura et al., 1985; Armstrong et al., 1988). In order to determine whether the eukaryotic receptor binding site and the ATP binding site might overlap, we examined the ability of ATP to affect pertussis toxin binding to red blood cells. ATP had no effect on toxin-induced hemagglutination, whereas fetuin was able to inhibit hemagglutination of red blood cells by pertussis toxin, presumably by mimicking the cellular receptor for the toxin. These results would suggest that ATP does not bind to the eukaryotic receptor site on the toxin but rather binds to a distinct site. However, fetuin did have a significant inhibitory effect on ATP binding to pertussis toxin. This suggests that the ATP binding site may be in a region of the molecule that is close enough to the receptor binding

site such that fetuin can partially interfere, perhaps sterically, with ATP binding to the toxin or that binding of fetuin to pertussis toxin may alter the conformation of the toxin such that it has a decreased affinity for ATP.

While the ATP binding site appears to be located on the B oligomer and the NAD binding site resides on the A subunit, we examined the effect of NAD, a substrate for pertussis toxin, on ATP binding in order to determine whether the ATP binding site might encompass part of the enzymatically active site. A precedent for such a model has been observed with diphtheria toxin, another ADP-ribosylating toxin that utilizes NAD as a substrate (Kandel et al., 1974; Lory & Collier, 1980; Lory et al., 1980). ApUp is believed to bind to a site that includes both a portion of the B moiety (the binding component) of diphtheria toxin and a portion of the NAD site on the enzymatically active moiety. We found that NAD had no effect on the binding of ATP to pertussis toxin, suggesting that the binding sites for ATP and NAD do not overlap on the pertussis toxin molecule.

Previous results indicate that ATP is required for manifestation of the enzymatic activity of pertussis toxin. The source of ATP for the toxin is unknown; however, the possibility exists that the eukaryotic cell provides this nucleotide to the toxin molecule. If that were the case, after binding of the B oligomer to the surface of the eukaryotic cell, a portion of the B oligomer might become exposed to the interior of the cell. ATP would then bind with high affinity to the toxin and promote the release of the A subunit thus activating the enzyme.

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Registry No. ATP, 56-65-5; GTP, 86-01-1; CTP, 65-47-4; TTP, 365-08-2; NAD, 53-84-9; ApUp, 1985-21-3; tripolyphosphate, 14127-68-5; pyrophosphate, 14000-31-8; phosphate, 14265-44-2; arsenate, 15584-04-0; sulfate, 14808-79-8.

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