Prior studies have described two functionally distinct ligand-binding sites on whole diphtheria toxin, the NAD site, which catalyzes the intracellular ADP-ribosylation reaction, and the P site, which affects toxin binding to sensitive cells. Occupancy of the P site by ATP or other phosphorylated compounds inhibits toxin attachment to cells. Here we show that binding of NAD site and P site ligands is competitive; and we characterize ligand-binding properties of two mutant forms of the toxin, CRM 45 and CRM 197. The data suggest that the NAD site, on the A moiety, lies immediately adjacent to the P site, formed by a strongly cationic region on the COOH-terminal half of B. The cationic character of the P site slightly alters the substrate specificity of the NAD site, and occupancy of either of the sites blocks ligand binding to the other. Possible roles of the P site in toxin attachment are discussed.

Although the enzymic activity which mediates toxicity of diphtheria toxin is well characterized, events which transpire between attachment of the toxin to the cell surface and the ADP-ribosylation of elongation factor 2 (EF-2) remain largely obscure. There is good evidence for the existence of specific cell surface receptors (1), and available data suggest that the receptor may correspond to a toxin-binding glycoprotein (Mr = 170,000) which has been isolated from guinea pig lymph node cells (2). It has been reported that toxin binds to lipid vesicles containing certain phospholipids, however, and it is possible that this may be significant in interactions with cell surfaces (3). All models of toxin attachment to the cell surface and transmembrane transfer of Fragment A remain largely speculative.

A potentially important clue to the mechanism of toxin attachment to the cell surface was provided by the discovery by Middlebrook and coworkers (4, 5) that nucleoside triphosphates are strong inhibitors of diphtheria toxin action on whole cells. These and certain other compounds containing multiple phosphate residues were found to interfere with binding of iodine-labeled toxin to cells. Corroborative results were reported by Chang and Neville (6), who demonstrated that ATP or βγ-methylene ATP inhibited toxin binding to isolated surface membranes from various animal sources.

We recently identified a site on diphtheria toxin (the P site) which binds ATP and other phosphate-containing compounds reported to inhibit toxin action (7). Correlations between inhibitory activity and affinity for the P site led us to propose that occupancy of this site might form the basis of the inhibition of toxin action reported by Middlebrook et al. Experiments performed independently by Proia et al. (8) had similar implications.

Evidence presented in the preceding article (9) suggested that the capacity of given toxin molecules to interact with NAD was related to their ability to bind ATP and other P site ligands. All preparations of whole toxin tested were shown to contain two fractions, which were separable on ATP-Sephrose. One of these (Fraction II) bound NAD or ATP with strong affinity, whereas the other (Fraction I) interacted only weakly with either nucleotide.

In the present work we have explored in greater detail possible relationships between the P site and the NAD-binding site on Fraction II of the toxin. The data indicate overlap between the two sites and suggest that the P site may be formed by a cationic region on B in close proximity to the NAD-binding site of the A moiety. Other results imply that the P site probably does not correspond to the receptor-binding site. A model incorporating these ideas is presented.

**EXPERIMENTAL PROCEDURES**

Most of the materials and methods used in this study are described in the preceding article (9). CRM 45 and CRM 197 were purified from culture fluids of C7 (β45)hm 723 and C7 (β197), respectively, as described by Pappenheimer et al. (10), except that DEAE-Sephadex (Pharmacia) was used for ion exchange chromatography. The ultraviolet absorption profile of purified CRM 197 was identical with that of Fraction II or with Fraction I that had been treated with 6 M urea and passed over Sephadex G-25 in the presence of 6 M urea.

**RESULTS**

**Correlations Between NAD Binding and ATP Binding to Diphtheria Toxin**—In flow dialysis measurements we found striking correlations between the binding of NAD and ATP to diphtheria toxin. Although the average number of binding sites per toxin molecule varied from preparation to preparation, we consistently found similar values for NAD and ATP (e.g. a typical preparation gave 0.34 ATP sites as compared with 0.31 sites for NAD). These results are consistent with findings reported elsewhere (7) that whole toxin may be separated into two fractions, one of which (Fraction II) binds either ATP or NAD with the same stoichiometry (1 mol per mol) and the other of which (Fraction I) is deficient in ATP or NAD binding and in NAD-dependent reactions.

* Studies reported elsewhere in this issue by Proia et al. (17, 18) correlate well with the results presented in this and the two accompanying articles (9, 15). We thank the authors of these studies for making their manuscripts available to us prior to submission for publication.
A competitive relationship between NAD site and P site ligands was determined by flow dialysis measurements on Fraction II. As shown in Fig. 1, binding of ATP to Fraction II was competitively inhibited by NAD ($K_i = 16.7 \mu M$) and NADH ($K_i = 10.5 \mu M$). Conversely, NAD binding was competitively inhibited by ATP, ADP, or AMP (Fig. 2). When ATP or NAD was included in toxicity assay reaction mixtures, the cytotoxic effects of Fraction I and Fraction II were reduced to the same extent (data not shown). At similar ligand concentrations, a greater degree of protection was observed for ATP, which may reflect preferential stability of this compound in cell culture.

Quenching of the native fluorescence of Fraction II by NAD provided an alternative means of demonstrating a competitive relationship between NAD and P site ligands. Highly phosphorylated compounds were shown to be more effective than those with low phosphate content in reversing NAD-induced quenching, and the relative effectiveness of the various compounds tested was the same as that found by flow dialysis.

Inhibitors of the NAD-Glycohydrolase Reaction Catalyzed by Fraction II—The effects of various inhibitors on the NAD-glycohydrolase activity of Fraction II were measured. As shown in Fig. 3 and Table I, there was good correlation between inhibition constants obtained in the NAD-glycohydrolase assay and those calculated from data on binding of labeled NAD to Fraction II. The extent of phosphorylation was again a primary determinant of binding, and there was an apparent lack of specificity with respect to the nucleoside moiety. Tri- and tetrapolyphosphate alone were excellent inhibitors.

This pattern of inhibition may be contrasted with that shown earlier for free Fragment A. For the fragment, adenine ($K_i = 30 \mu M$) was the most potent inhibitor of the NAD-glycohydrolase activity; linkage of ribose and increasing numbers of phosphate residues to adenine progressively decreased its affinity. Direct measurements of binding by flow dialysis gave similar results. We recently reconfirmed that ATP inhibited Fragment A-catalyzed NAD-glycohydrolase activity only weakly ($K_i > 2 \mu M$) and demonstrated that neither inositol hexaphosphate nor tetrapolyphosphate had detectable inhibitory activity at concentrations as high as 500 $\mu M$ (data not shown).

Potential degradation of [α-32P]ATP by toxin was measured in mixtures containing 100 $\mu M$ toxin and 50 $\mu M$ ligand during incubations of up to 2 h at 4°C. No alterations in the ATP were detectable by chromatography of samples on polyethyl- eneimine. This result correlates with the fact that the affinity for toxin of the nonhydrolyzable ATP analog $\beta,\gamma$-methylene ATP is comparable to that of ATP.

Inhibition of the Auto-ADP-Ribosylation Reaction by ATP—It was shown in the preceding article that Fraction II catalyzed transfer of ADP-ribose to itself (auto-ADP-ribosylation), whereas Fraction I was only weakly active in this regard (9). An analysis of initial rates (Fig. 4) indicated a $K_i$ for NAD of 11 $\mu M$, correlating this reaction with the NAD site previously characterized (9, 11).

ATP competitively inhibited the auto-ADP-ribosylation of Fraction II with a $K_i$, 10 $\mu M$, very close to that seen for this ligand in the NAD-glycohydrolase reaction (8.1 $\mu M$) (9). When auto-ADP-ribosylated Fraction II was passed through Sepharose-4B, about 1% of the radioactivity was in the 280 kDa form, 5% in the 130 kDa form, and 94% in the 15 kDa form.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>NAD-glycohydrolase $K_i$ (μM)</th>
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<td>ATP</td>
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<td>8.2</td>
</tr>
<tr>
<td>Tetrapolyphosphate</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
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<td>nd*</td>
</tr>
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</tr>
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</tr>
<tr>
<td>ADP-ribose</td>
<td>520</td>
<td>nd*</td>
</tr>
</tbody>
</table>

* Not determined.

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Fig. 1 (left). Effect of NAD and NADH on ATP binding to Fraction II of diphtheria toxin. Measurements of ATP binding were performed by flow dialysis using [α-32P]ATP. The flow cell upper chamber contained 82 $\mu M$ Fraction II and 0.045 $\mu M$ [32P]ATP in 50 mM Tris-HCl buffer, pH 7.2 (total volume, 255 μl). Tris-HCl buffer was pumped through the lower chamber at a rate of 3 ml per min, and 1-ml fractions were collected. Unlabeled ATP was added to the upper chamber in 1-μl aliquots every fifth fraction. Radioactivity was determined by CsI-nal counting in toluene-ethanol. The flow cell contained 82 $\mu M$ Fraction II. The concentrations of inhibitors used were: ●, uninhibited reaction; ○, ATP (48 μM); ■, ADP (68 μM); ▲, AMP (136 μM).

Fig. 2 (center). Inhibition of NAD binding to Fraction II by ATP, ADP, and AMP. Flow dialysis was performed with [32P]NAD essentially as described in the legend to Fig. 1. The concentrations of inhibitors used were: ●, no inhibitor; □, ATP (56 $\mu M$); △, ADP (42 $\mu M$); ■, AMP (51 $\mu M$); ▲, adenine (30 $\mu M$). Rates of NAD hydrolysis (v) are expressed in terms of picomoles of NAD hydrolyzed/h.
NAD-glycohydrolase activity was detected at concentrations hydrolysis of NAD at a rate somewhat slower than that. This result absorbed onto the column; elution with 0.5 M NaCl removed graphed on ATP-Sepharose (Fig. 5), none of the radioactivity included the lability of isolated B, uncertainty about the identity of the cell surface receptor, and difficulties inherent in studying cell surface attachment and penetration phenomena.

In the present report we have examined relationships between two functionally distinct sites on whole diphtheria toxin. One, the NAD site, is involved in the intracellular ADP-ribosylation event; the other, the P site, apparently affects the process by which the toxin attaches to the cell surface. The properties of the NAD on whole toxin leave little doubt that this site corresponds to the NAD site previously described on free Fragment A (11). The P site, because of its function and the fact that free Fragment A contains no site with corresponding properties, has been assumed to be located on the B moiety. A direct test for the presence of the P site on isolated B has been hampered by the relatively low solubility of the isolated fragment, but the indirect evidence available is basically consistent with this location. Results presented here demonstrate a strong interaction between the P site and the NAD site on the A moiety. We were intrigued initially by the apparent correlation in binding of ATP and NAD to the same fraction of diphtheria toxin, Fraction II. Further evidence that the NAD site and the P site were related came from studies showing competition for NAD site were related came from studies showing competition for NAD and P site ligands. Competition for NAD and P sites is illustrated in Fig. 7. The desalted protein up to 5 μM was chromatographed on ATP-Sepharose (Fig. 5), none of the radioactivity was removed by gel filtration on a column (0.6 x 20 cm) of Sephadex G-25 equilibrated with 50 mM Tris-HCl (pH 7.2) at 4°C. The desalted sample was applied to a 1-ml column of ATP-Sepharose equilibrated with the same buffer and chromatographed at about 4 ml/h. Each fraction (0.5 ml) was examined for radioactivity (0) and absorbance at 280 nm (0). At the arrow, 0.5 M NaCl in equilibration buffer was applied to the column, and monitoring was continued.

Fig. 4 (left). Effect of ATP on the auto-ADP-ribosylation reaction catalyzed by Fraction II. Toxin (20 μM) in 50 mM Tris-HCl, pH 9.5, was incubated with 5 to 200 μM [32P]NAD in the presence (0) or absence (0) of 10 μM ATP for 20 min at 37°C. Duplicate samples were then removed and spotted onto filter paper impregnated with 12% trichloroacetic acid and 0.1 M phosphoric acid. The filters were washed repeatedly with 5% trichloroacetic acid, 40 mM phosphoric acid, and then dried and counted. Identical reaction mixtures containing [carbonyl-14C]NAD were examined to ensure that less than 10% of the substrate was hydrolyzed during the incubation period. Velocity (1) is expressed as moles of ADP-ribose bound/mol of Fraction II/h.

Fig. 5 (center). Affinity chromatography of auto-ADP-ribosylated Fraction II on ATP-Sepharose. Toxin (20 μM) was incubated with 100 μM [32P]NAD in 50 mM Tris-HCl, pH 9.5, for 3 h at 37°C (total volume, 150 μl). Unbound ADP-ribose and nicotinamide were removed by gel filtration on a column (0.6 x 20 cm) of Sephadex G-25 equilibrated with 50 mM Tris-HCl (pH 7.2) at 4°C. The desalted sample was applied to a 1-ml column of ATP-Sepharose equilibrated with the same buffer and chromatographed at about 4 ml/h. Each fraction (0.5 ml) was examined for radioactivity (0) and absorbance at 280 nm (0). At the arrow, 0.5 M NaCl in equilibration buffer was applied to the column, and monitoring was continued.

Fig. 6 (right). NAD-glycohydrolase activity of CRM 45 and CRM 197. NAD hydrolysis was measured in the presence of 1.5 μM Fraction II (0), CRM 45 (0), or CRM 197 (0), as described in Fig. 3. The respective closed symbols represent reaction mixtures containing, in addition to the above, 40 μM ATP. Less than 5% of the substrate was hydrolyzed during the incubation period.

adex G-25 to remove unbound ligand and then chromatographed on ATP-Sepharose (Fig. 5), none of the radioactivity absorbed onto the column; elution with 0.5 M NaCl removed only unmodified toxin. Thus self modification of toxin by NAD blocked the toxin’s ability to interact with ATP.

Interaction of NAD with Mutant Forms of Diphtheria Toxin—Several mutant forms of diphtheria toxin have been isolated, and their defects have been localized to Fragment A, Fragment B, or both (12). We have examined two of these proteins for ligand binding and NAD-glycohydrolase activity. CRM 45, a chain termination fragment (M, 45,000) containing Fragment A plus about half the B moiety, catalyzed the hydrolysis of NAD at a rate somewhat slower than that observed with diphtheria toxin Fraction II (Fig. 6). The reaction catalyzed by CRM 45, like the Fragment A-catalyzed reaction, was not inhibited by ATP. For the second mutant protein, CRM 197, which possesses a defective A fragment, no NAD-glycohydrolase activity was detected at concentrations of the protein up to 5 μM incubations for 2 h at 37°C. This result is consistent with the findings of Michel and Dirkx (13), showing no quenching of CRM 197 fluorescence by NAD. We also found that Fragment A from CRM 197 failed to interact with NAD, as evidenced by the absence of NAD-glycohydrolase activity and the inability of NAD to quench protein fluorescence. We were unable to detect binding of ATP to either CRM 45 or CRM 197 by flow dialysis, and neither CRM 45 nor CRM 197 was retained by ATP-Sepharose. No binding of NAD to CRM 197 was found by flow dialysis. Neither CRM 45 nor CRM 197 exhibited significant auto-ADP-ribosylation activity.

Discussion

The principal barriers remaining to our understanding the action of diphtheria toxin (and other toxins known to act catalytically on cytosolic targets) concern mechanisms of toxin attachment to the cell surface and penetration of the catalytic center to the cytosol. Whereas our understanding of structure and activity in the A moiety is relatively advanced, a combination of factors has hampered studies of Fragment B. These include the lability of isolated B, uncertainty about the identity of the cell surface receptor, and difficulties inherent in studying cell surface attachment and penetration phenomena.

In the present report we have examined relationships between two functionally distinct sites on whole diphtheria toxin. One, the NAD site, is involved in the intracellular ADP-ribosylation event; the other, the P site, apparently affects the process by which the toxin attaches to the cell surface. The properties of the NAD site on whole toxin leave little doubt that this site corresponds to the NAD site previously described on free Fragment A (11). The P site, because of its function and the fact that free Fragment A contains no site with corresponding properties, has been assumed to be located on the B moiety. A direct test for the presence of the P site on isolated B has been hampered by the relatively low solubility of the isolated fragment, but the indirect evidence available is basically consistent with this location.

Results presented here demonstrate a strong interaction between the P site and the NAD site on the A moiety. We were intrigued initially by the apparent correlation in binding of ATP and NAD to the same fraction of diphtheria toxin, Fraction II. Further evidence that the NAD site and the P site were related came from studies showing competition between NAD and P site ligands. Competition for NAD binding was demonstrated by flow dialysis, inhibition of NAD-glycohydrolase and auto-ADP-ribosylation activities, and reversal of NAD-induced quenching of toxin fluorescence. Inhibition of ATP binding by NAD and other compounds was shown by flow dialysis.

Although other more complex models could be formulated, the simplest interpretation of our data would be that the NAD site on the A moiety overlaps the P site in such a manner that occupancy of either site blocks access of ligands to the other. A possible arrangement of various binding sites on diphtheria toxin is illustrated in Fig. 7.
We have placed the NAD site on the A moiety adjacent to a cationic region on the COOH-terminal half of the B moiety, which forms the P site. The fact that neither Fragment A nor CRM 45 has significant affinity for P site ligands suggests that the P site is located on the COOH-terminal third of the B moiety, i.e., that portion of B missing from CRM 45. The ligand specificity of the P site implies that this site is formed by a region of dense positive charge and that shape constraints on P site ligands are minor (7). Pappenheimer has proposed from results obtained by isoelectric focusing that the region of toxin missing from CRM 45 bears a net positive charge (14). The proximity of such a cationic region to the NAD site would also be consistent with the finding that in the auto-ADP-ribosylation reaction, the major portion of covalently bound label attaches to the B moiety (9). Finally, the model is in agreement with results presented in the accompanying article (15), showing that when ATP in the P site is photolyzed, linkage occurs to the A moiety.

Data obtained with CRM 197 are also consistent with the model, in that a genetic alteration of the A moiety which affects NAD binding and NAD-dependent reactions apparently also blocks interaction with P site ligands. The fact that the ultraviolet absorbance profile of CRM 197 was identical with that of Fraction II negates the possibility that access to the P site might be blocked by a tightly bound, low molecular weight, ultraviolet-absorbing compound, such as that found on Fraction I (7). In initial studies of ATP binding to Fraction II we postulated that the P site might correspond to the receptor-binding site on the B moiety (7). The fact that CRM 197 binds neither ATP nor NAD now leads us to doubt this. Since the B moiety of CRM 197 is apparently fully functional in binding to and blocking cell surface receptors, we would have expected the P site to have been accessible, had our initial postulate been true.

The mechanism by which occupancy of the P site blocks attachment of toxin to cells remains uncertain. If our conclusion is correct, that the P site does not correspond to the receptor-binding site, then binding of P site ligands may alter the receptor-binding site by allosteric mechanisms. The fact that CRM 45 is deficient in receptor binding implies that this function, like the P site, resides on the COOH-terminal region of B. Alternatively, it may be that binding of toxin to cell surfaces occurs by a complex sequence of events, one of which involves the P site. Thus, for example, one may envision a model in which reversible binding to receptors is followed by interaction of the P site with phospholipid(s) or other negatively charged cell surface components. If this were the case, then ATP or other P site ligands might competitively inhibit the latter event.

The presence of a hydrophobic, Triton-binding domain within the NH₂-terminal region of B has led to speculation that the B moiety may become inserted into the lipid bilayer, following initial reversible attachment of the toxin to the appropriate receptor (16). Although there is no definitive evidence that this occurs, an intermediate event involving interaction of a site such as the P site with hydrophilic head groups of phospholipids would seem quite possible. With information from various systems now under study in several laboratories, it should be possible to subject models such as this to experimental test within the near future.

Acknowledgment—We are grateful to Patricia D. Bernard for excellent technical assistance.

REFERENCES