Interaction of Fragment A from Diphtheria Toxin with Nicotinamide Adenine Dinucleotide*

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SUMMARY

The inhibition of protein synthesis by diphtheria toxin in extracts of mammalian cells results from the inactivation of elongation factor 2 (EF-2) by covalent attachment of the adenosine diphosphate ribose (ADPR) moiety of NAD+.

\[ \text{NAD}^+ + \text{EF-2} \rightleftharpoons \text{ADPR-} \text{EF-2} + \text{nicotinamide} + \text{H}^+ \]

This reaction is catalyzed by Fragment A (mol wt 24,000) or other less common fragments generated by limited proteolysis and reduction of the toxin, but not by the toxin itself (mol wt 63,000). This report describes studies of the interaction of \( \text{NAD}^+ \) with Fragment A.

In addition to its major enzymic activity, Fragment A also catalyzes the slow hydrolysis of the nicotinamide-ribose linkage of NAD+.

\[ \text{NAD}^+ + \text{H}_2\text{O} \rightleftharpoons \text{ADPR} + \text{nicotinamide} + \text{H}^+ \]

This activity (NAD+glycohydrolase; EC 3.2.2.5) is several orders of magnitude lower than the \( \text{NAD}^+:\text{EF-2-ADPR-} \) transferase activity and probably does not contribute to the toxicity of diphtheria toxin. However, its existence implies a direct binding of \( \text{NAD}^+ \) to Fragment A and is of interest with regard to the mechanism of transfer of ADPR to EF-2.

Binding of \( \text{NAD}^+ \) and related compounds to Fragment A was studied by dynamic dialysis, fluorescence quenching, and spectral absorbance techniques. The fragment contains a single binding site for a \( K_D \) of about 8 \( \mu \text{M} \). Binding of \( \text{NAD}^+ \) is rapidly reversible, and no evidence of a covalent ADPR-Fragment A intermediate was found. \( \text{NAD}^+ \) strongly quenches the intrinsic fluorescence of the fragment (emission maximum 333 nm) and induces a broad peak of absorbance at 360 nm (\( \epsilon \approx 500 \)). Both effects are interpreted to result from formation of a charge transfer complex between the nicotinamide moiety of \( \text{NAD}^+ \) and at least one of the tryptophan residues of the fragment. Binding of \( \text{NAD}^+ \) also enhances the resistance of the fragment to trypsin or chymotrypsin. No significant change in either the sedimentation coefficient (2.19 S) or the rotational relaxation time was found upon addition of \( \text{NAD}^+ \).

The affinities of various \( \text{NAD}^+ \) analogs and partial structures for the binding site on Fragment A closely parallelled their activities as inhibitors or substrates of ADPR transfer. These results, together with the fact that Fragment A alone hydrolyzes the same linkage which is ruptured during ADPR transfer, leave little doubt that it is this binding site which is involved in transfer of ADPK to EF-2. A model of the ADP-ribosylation of EF-2 is proposed based on these and other results.

Inhibition of protein synthesis by diphtheria toxin in mammalian cells and cell-free systems is believed to result from inactivation of the peptidyl-tRNA translocation factor, EF-2.\(^1\) The factor, a protein of molecular weight about 100,000 (\( I \)), is inactivated by a modification involving covalent attachment of the adenosine diphosphate ribose moiety (ADPR) of \( \text{NAD}^+ \) (2-4).

\[ \text{NAD}^+ + \text{EF-2} \rightleftharpoons \text{ADPR-} \text{EF-2} + \text{nicotinamide} + \text{H}^+ \]

Results from our laboratory (5, 6) and another (7, 8) have shown that it is not the whole toxin molecule but rather a fragment thereof which is the catalytic entity in this reaction. The toxin is apparently synthesized as an intact 63,000-dalton polypeptide which is toxic for animals but enzymically inactive. For enzymic activity to be expressed, the toxin must be exposed to limited proteolysis, for example, with trypsin, and subsequently reduced. Limited treatment with trypsin cleaves the toxin at three or more closely spaced sites (9, 10) resulting in the formation of two major fragments, A (mol wt 24,000) and B (mol wt 39,000), linked by a single disulfide bridge. Upon reduction of this bridge, the fragments may be dissociated, and the liberated Fragment A can be shown to be enzymically active. No activity has been shown for Fragment B, although it is presumed to be required for attachment to or entry into sensitive cells inasmuch as Fragment A alone is not toxic.

\(^1\) The abbreviations used are: EF-2, elongation factor 2, formerly termed aminoacyltransferase 2; ADPR, adenosine diphosphate ribose; SLS, sodium dodecyl sulfate; DNS, dansyl, 5-di-methylamino-1-naphthalenesulfonate.
These results suggest that both proteolysis and reduction may be essential in the expression of toxic activity in cells. Other enzymatically active fragments besides Fragment A have been found in certain toxin preparations, but these are rare, and with one possible exception (11), all are larger than and contain Fragment A (7, 8). Thus Fragment A may be the predominant enzymatically active fragment generated in vivo, and hence may be responsible for inactivation of EF-2 in whole cells.

Here we report studies on the interaction of NAD⁺ and related compounds with Fragment A. Portions of this work have been reported in abstract form (12, 13).

**EXPERIMENTAL PROCEDURES**

**Reagents**

The sources of the purchased materials used in these studies were as follows: disopropyl phosphorofluoridate-treated carboxypeptidases A and B, t-1-oxalylamido-2-phenylthiazolyl ketone-treated trypsin, ethyltrypsin, and ribonuclease from Worthington; pyridine nucleotides, nucleosides, and bases from Sigma; acrylamide gel reagents from Bio-Rad. All other chemicals were of the highest grade available commercially.

**Radioactive NAD⁺**

NAD⁺ labeled with ¹⁴C in the nicotinamide moiety (50 μCi per pmole) was purchased from Amersham-Searle. NAD⁺ labeled uniformly with ¹³C in the adenosine moiety was prepared enzymically from [¹³C]ATP and NAD⁺ purified according to methods previously described (5). The specific activity of the final product was approximately 419 μCi per pmole.

**Fragment A**

Fragment A was derived from purified toxin which had been treated with trypsin in order to convert all intact toxin to the nicked form (6). Reduction of the nicked protein was by two methods: (a) in the presence of 0.1 M dithiothreitol for 90 min at 37°, or (b) in the presence of 0.5 M guanidine HCl and 0.1 M dithiothreitol for 120 min at 25°. In both cases, the precipitate was removed by centrifugation and the supernatant containing Fragment A was dialyzed overnight against 50 volumes of 0.1 M 2-hydroxyethylisulide in buffer to block free sulfydryl groups. Fragment A was then purified in one step by chromatography on a column of Sephadex G 100 (5.0 x 90 cm) equilibrated with 50 mM Tris-HCl, pH 8.2.

Recently we have found it more satisfactory to perform the reduction and chromatography in the presence of 4 M urea containing 50 mM 2-mercaptoethanol. In this case the Fragment A was collected from the Sephadex column and, after dialysis against 0.1 M 2-hydroxyethylisulide and concentration by ultrafiltration, was rechromatographed on Sephadex G-100 equilibrated with 50 mM Tris-HCl, pH 8.2, containing 0.1 mM EDTA.

**Assay of ADP-Ribosylation Activity**

The enzyme activity of Fragment A was assayed by measuring incorporation of radioactivity from [¹³C]NAD⁺ labeled uniformly with ¹³C in the adenosine moiety into trichloroacetic acid-precipitable material in the presence of partially purified EF-2 from rabbit reticulocytes. Except where indicated, the materials employed and the conditions of assay were identical with those described previously (6).

**NAD⁺-Glycohydrolase Activity**

The hydrolysis of NAD⁺ was determined under two different sets of conditions. System A was used to measure substrate specificity of NAD⁺ analogs. System B was employed for routine assay and for measurement of inhibition by NAD⁺-related compounds.

**System A**—Unless otherwise indicated, each sample contained NAD⁺ or its analog (100 μM) and Fragment A (50 μM) in 50 mM sodium phosphate buffer, pH 7.8 (final volume 0.52 ml). Samples were incubated at 37° for periods up to 5 hours. Aliquots of 100 μl were removed at intervals and mixed immediately with 1.5 ml of 1 M KCN. The concentration of the cyanide addition product of NAD⁺ was determined by measurement of fluorescence (excitation, 345 nm; emission, 435 nm) in a Farrand Mark 1 spectrofluorometer.

**System B**—Each sample (0.2 ml) contained [nicotinamide-¹⁴C]-NAD⁺ (1 to 6 μM) and Fragment A (0.25 μM) and the inhibitor being tested in 50 mM sodium phosphate buffer, pH 7.8. Samples were incubated at 37° for 60 min and the reaction was stopped by the addition of 0.05 ml of 5 M KCN. Each sample was then extracted with 2.0 ml of water-saturated reagent grade ethyl acetate. Samples were evaporated under nitrogen, and the residues were then mixed with 10 ml of scintillation fluid (25% Triton X in toluene, 0.7% 2,5-di-phenyloxazole [POPOP], and 0.005% 1,4-bis[2-(5-phenyloxazoly)-benzene (POPOP)) and counted in a Beckman scintillation counter. Greater than 95% of the nicotinamide and about 2% of the NAD⁺ was extracted into the organic phase.

**Dynamic Dialysis**

Binding of NAD⁺ was measured at 4° by the dynamic dialysis technique of Colowick and Womack (14). The Lucite apparatus employed was identical with that described except that the depth of the lower chamber was only 5 mm and buffer exited the lower chamber through a vertical channel in the center which intersected a horizontal channel leading to the exterior. Fragment A, 100 nmoles in 50 mM sodium phosphate buffer, pH 7.8, was placed in the lower chamber and 50 nmoles in 1 M KCN to bring the volume to 1.5 ml. Unlabeled NAD⁺ was added in 5-μl portions at 5-min intervals. Sodium phosphate buffer was passed through the lower chamber at a rate of 2.7 ml per min. Samples of 1 ml were collected and mixed with 17 ml of scintillation fluid (25% Triton X in toluene, 0.7% 2,5-diphenyloxazole, and 0.005% 1,4-bis[2-(5-phenyloxazoly)-benzene and counted.

The binding of NAD⁺ was measured in two ways. The first involved measurement of the competitive inhibition binding of unlabeled, commercial NADH as described above. In the second, adenine-labeled NAD⁺ was first reduced with alcohol dehydrogenase (4 μg per ml) in the presence of 50 mM Tris HCl buffer, pH 8.8, and 0.2 M ethanol, at 25° for 1 hour, and the binding was measured directly, with titration by unlabeled NADH reduced in the same manner. Both ethanol and alcohol dehydrogenase were present in the upper chamber, and ethanol was included in the buffer flushed through the lower chamber. Controls showed that ethanol at the concentration employed had no effect on the binding of NAD⁺.

**Fluorescence Measurements**

Titrations were carried out in a Farrand Mark 1 spectrofluorometer, modified to contain a thermostatically controlled cuvette holder. Temperature was controlled to within 0.1°. Titrations were performed by adding 5- or 10-μl increments of NAD⁺, analog, or derivative in 50 mM Tris-HCl, pH 8.2, to a 1 ml of Fragment A, 1 μM, in buffer. Samples were stirred after each addition and the decline in protein fluorescence at 333 nm ( excitation 285 nm) was measured. Samples were kept at 25° and shielded from incident light when readings were not being taken. The fluorescence of samples was compared with that of a 1% solution of tryptophan of comparable initial fluorescence intensity. To correct for attenuation of the excitation beam by added ligand, tryptophan solutions were similarly titrated and appropriate corrections were made. All data were corrected for dilution.

**Polarization of Fluorescence**

Fragment A was labeled in the presence of saturating concentrations of NAD⁺ according to the method of Rinderknecht (15) using DNS on Celite 10%. The presence of NAD⁺ was found to prevent conjugation of the dye to groups having internal molecular rotation. The number of dansyl groups per molecule of protein was determined by measurement of the optical density of 280 nm (for the protein) and 340 nm (DNS) using 4200 as the molar extinction coefficient of the dye (16). Polarization of fluorescence measurements were conducted in the Farrand spectrofluorometer, using polarizing filters in the ex-
cition and emission beams. Measurements were conducted on DNS-Fragment A (1 μm) in 50 mM Tris-HCl, pH 8.2, in the presence or absence of 200 μM NAD+. The vertically and horizontally polarized components of the fluorescent light were recorded at temperatures between 8 and 35°C. Excitation was at 345 nm and emission at 525 nm.

Differential Sedimentation

Differential sedimentation was performed according to the procedure of Schumaker and Adams (17). A 1° negative wedge was used to separate the schlieren patterns of the experimental and reference cell. Fragment A, 4 to 10 μg per ml in 0.1 M Tris-HCl, pH 8.2, 0.15 M KCl, was centrifuged at 59,780 rpm for 3 hours at 20°C. Ribonuclease, 7 μg/ml, was used as a reference protein. Photographs were taken at 16-min intervals and the plates were read on a Nikon micro-comparator. Computations were performed as described by Schumaker and Adams (17).

Difference Spectra

Difference spectra were taken employing pairs of matching cylindrical tandem double cells (1-cm light path). NAD+ was added in microliter quantities to Fragment A and to the buffer in the reference cuvette. An equal volume of buffer was added to the protein reference cuvette. Difference spectra were recorded immediately on a Cary model 15 spectrophotometer.

Paper Chromatography

Ascending paper chromatography was carried out at room temperature for 30 hours on Whatman No. 3MM paper in a solvent system of isobutyric acid-ammonia-water (66:1:33). The paper was sliced into strips which were then cut into 30 fractions, 2 X 1 cm, and each fraction was soaked for 30 min in 1 ml of water in a scintillation vial. The fractions were then counted in 17 ml of scintillation fluid.

Polyacrylamide Gel Electrophoresis

The gels used contained 10% acrylamide and 0.27% methylene-bisacrylamide in phosphate buffer, 0.1% in sodium dodecyl sulfate. The methods of preparation of gels and samples and conditions for electrophoresis were as described previously (5). Analytical disc gel electrophoresis was also performed under nondenaturing conditions as described by Maizel (18). The gels were stained overnight in 0.25% Coomassie brilliant blue in 10% trichloroacetic acid followed by destaining by diffusion in 7% acetic acid.

RESULTS

Heterogeneity of Fragment A—Purified Fragment A migrated as a single band of molecular weight 24,000 in SDS-polyacryl-
amide gels, whereas three closely spaced, major bands were observed in 7.5% analytical disc gels in the absence of detergent. All three bands were enzymatically active (Fig. 3). Recent evidence from work on the primary structure of Fragment A suggests that the bands may correspond to three major forms which differ in their COOH termini. Thus from the BrCN digest of the fragment, three different COOH-terminal peptides have been isolated, the partial sequences of which are, -Asn-Arg, -Asn-Arg-Val-Arg, and -Asn-Arg-Val-Arg-Arg.2 The charge differences due to the variation in the number of basic amino acids presumably account for the differences in electrophoretic mobility.

The simplest explanation for the heterogeneity at the COOH terminus of Fragment A is that it is generated by nonspecific attack by trypsin at any of 3 closely spaced arginines within intact toxin. The fact that Fragment A is derived from the NH2 terminus of intact toxin, which has been proven by sequence homology (19), is consistent with this. It is possible, in fact, that there are more than 3 basic residues in the trypsin-sensitive region, inasmuch as the terminal arginine of the longest form of Fragment A may not be linked directly to the NH2 terminus of Fragment B within intact toxin.

1 R. Drazin, R. J. DeLange, and R. J. Collier, unpublished data.

![Fig. 1](link) (left). Hydrolysis of NAD+ by Fragment A. NAD+, 100 μM in 50 mM Tris-HCl, pH 8.2, was incubated at 35°C for periods up to 2 hours in the absence of (Ο—Ο) and presence (Ο—Ο) of 25 μM of Fragment A. At the times indicated, samples of 0.15 ml were removed and mixed with 1.5 ml of 1 M KCN. The concentration of the cyanide addition product of NAD+ was measured by fluorescence with excitation at 345 nm; emission at 435 nm.

![Fig. 2](link) (right). Paper chromatography of the products of NAD+ hydrolysis. The reaction was conducted in a mixture (0.13 ml) containing 5.6 nmoles of sodium phosphate buffer, pH 7.8, and [3H]NAD+ labeled in either the nicotinamide (NIC) moiety (200 μM) or the adenosine moiety (2.5 μM) in the presence or absence of 2.6 nmoles of Fragment A. After incubation for 4 hours at 37°C, 10-μl portions of the reaction mixtures were spotted on Whatman No. 3MM paper. Unlabeled markers, AMP, ADP, NAD+, NMN, and nicotinamide were run on the same paper. Ascending chromatography was performed as described under "Experimental Procedures." A, adenosine-labeled NAD+; B, adenosine-labeled NAD+ plus Fragment A; C, nicotinamide-labeled NAD+; D, nicotinamide-labeled NAD+ plus Fragment A.

NAD+-Glycohydrolase Activity of Fragment A—When NAD+ was incubated with high concentrations of Fragment A at 25°C in the absence of EF-2, there was a gradual disappearance of the pyridine nucleotide as measured by its capacity to form a fluorescent addition product from KCN (Fig. 1). Difference spectra revealed the concomitant formation of absorbance peaks at 258, 266, and 276 nm, which are characteristic of free nicotinamide.

[3H]NAD+ labeled in either the nicotinamide or adenosine moiety was incubated in the presence or absence of Fragment A, and the reaction products were chromatographed on paper together with unlabeled markers. As shown in Fig. 2, after incubation with Fragment A, the labels migrated identically with the adenosine and ADPR. In other experiments a 10-fold molar excess of NAD+ was shown to be hydrolyzed completely by the fragment. Hence, Fragment A has NAD+-glycohydrolase activity.

NAD+ + H2O — ADPR + nicotinamide + H+.

Although this activity is lower by several orders of magnitude than the NAD+:EP-2-ADPR-transferase activity, it is certain that the hydrolase activity is in fact due to Fragment A and not a contaminant. This is shown unequivocally by the correlation of the profiles of the two activities eluted from polyacrylamide gels containing Fragment A. As shown in Fig. 3, both profiles have three peaks which correspond to the three major electrophoretic forms of Fragment A. In agreement with this conclusion, the NAD+-glycohydrolase activity, like the major activity
of Fragment A, was essentially unchanged after incubation of the fragment at 100° for 30 min at pH 8.0 or at 25° for 1 hour at pH 1 or 12. Also, upon treatment of toxin or trypsin-treated toxin with thiol, a marked increase in NAD\textsuperscript+ glycohydrolase activity occurred which correlated with the amount of Fragment A released (Table 1). The specific activity of toxin or trypsin-treated toxin was no greater than 10% that of Fragment A on a molar basis.

**Table 1**

**Effect of reduction on NAD\textsuperscript+-glycohydrolase activity of toxin and trypsin-treated toxin**

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Fragment A, relative concentration (pM)</th>
<th>NAD\textsuperscript+ hydrolysis, relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxin</td>
<td>120</td>
<td>3</td>
</tr>
<tr>
<td>Toxin, reduced</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>Toxin, trypsin-treated</td>
<td>55</td>
<td>4</td>
</tr>
<tr>
<td>Toxin, trypsin-treated, reduced</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Pure Fragment A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. NAD\textsuperscript+-glycohydrolase and NAD\textsuperscript+:EF-2-ADPR-transferase activities of Fragment A eluted from nondenaturing polyacrylamide gels. Samples of Fragment A (100 µg) were subjected to electrophoresis in 18 cm long 10% polyacrylamide gels (18). The gels were sliced into 2 mm thick fractions, and each was eluted overnight at room temperature with 0.1 ml of 50 mM Tris-HCl, pH 8.2, containing 1 mM EDTA. A 25 µl portion from each fraction was assayed for NAD\textsuperscript+-glycohydrolase activity according to Method B, except that the incubation time was 2 hours and 10 µg of crystalline bovine serum albumin were added to each assay mixture. Amounts equivalent to 0.25 µl (5 µl of a 1:20 dilution) from each fraction were assayed for ADP-ribosylation activity as reported (3) except that the assay mixture contained 60 pmoles of radioactive NAD\textsuperscript+ and 20 pmoles of EF-2. Fractions 1 to 29 and 61 to 90 showed only background activities in both assays.

Fig. 4. Dynamic dialysis measurements of binding of NAD\textsuperscript+ to Fragment A. Top frame, Fragment A, 100 µg in 50 mM sodium phosphate buffer, pH 7.8, was mixed with [\textsuperscript{i}C\textsuperscript{14}]NAD\textsuperscript+ in a final volume of 1.5 ml in the upper chamber of the apparatus as described under “Experimental Procedures.” Where indicated by arrows, unlabeled NAD\textsuperscript+ was added to bring the final concentrations to those shown. A final addition of NAD\textsuperscript+ to 13 mM released essentially all unlabeled NAD\textsuperscript+ from the fragment. Bottom frame, Scatchard plot of the average of data from five experiments as in top frame. From the y axis intercept, there are 1.01 binding sites for NAD\textsuperscript+ per protein molecule of 24,000 molecular weight; from the slope, $K_D = 8.3 \mu M$. The bars indicate the range of values.

The turnover number of the fragment in the NAD\textsuperscript+-glycohydrolase reaction was about 0.05 mole of NAD\textsuperscript+ per min per mole of the fragment at the optimal temperature, 37°. At 25°, which is the temperature optimum for transfer of ADPR to EF-2, the rate of NAD\textsuperscript+ hydrolysis was about half that at 37°. The pH optimum was about 8.0 in either Tris-HCl or sodium phosphate buffer, which correlates well with the pH optimum of ADPR transfer to EF-2 (pH 8.2 to 8.5).

Measurement of NAD\textsuperscript+ Binding by Dynamic Dialysis—The hydrolytic of NAD\textsuperscript+ by Fragment A described above was sufficiently rapid to prevent us from using equilibrium dialysis to measure binding of the pyridine nucleotide to the fragment. However, the dynamic dialysis technique of Colowick and Womack (14) was employed with success. This technique involved measurement of the rate of diffusion of labeled NAD\textsuperscript+ across a dialysis membrane from mixtures of NAD\textsuperscript+ and Fragment A. Sufficient data for a complete binding curve could be obtained within less than an hour, during which time less than 3% of the NAD\textsuperscript+ was hydrolyzed. Fig. 4 shows the data from a typical dynamic dialysis experiment and a Scatchard plot of the average results of five such experiments. The results indicate that Fragment A contains one NAD\textsuperscript+ binding site (1.01 ± 0.09), with a dissociation constant of 8.3 ± 2 µM. The linearity of this plot indicates there is little if any variation of $K_D$ among the three electrophoretic forms of Fragment A.

Quenching of Protein Fluorescence by NAD\textsuperscript+—As shown in Fig. 5, the intensity of fluorescence of Fragment A decreased markedly as the concentration of NAD\textsuperscript+ was increased. At saturating concentrations of NAD\textsuperscript+ the fluorescence (excitation, 285 nm; emission, 333 nm) was reduced to a level of about 20 to

The specific activity of toxin or trypsin-treated toxin was no greater than 10% that of Fragment A on a molar basis.
A contains 2 tryptophan residues, while we have obtained a pound indolylethylnicotinamide. The fluorescence of the indolyl ring is entirely quenched in the compound, and a long either indole or nicotinamide alone. Presumably, this is also a side chain of tryptophan in the protein. This interpretation of Fragment A. Michel et al. (19) have reported that Fragment A intermediate was obtained. NAD+ binding is rapidly reversible and mediate was obtained. NAD+ binding is rapidly reversible and reversible.

Alterations in Spectral Absorbance upon Binding of NAD+—The ultraviolet difference spectrum of Fragment A generated upon binding of NAD+ showed peaks near 281 nm for tyrosine and 294 nm for tryptophan. Both indicated a red shift presumably due to transfer of certain residues to a more hydrophobic environment. A more interesting change was observed farther toward the visible region. As shown in Fig. 6, a new absorption band was produced in the presence of NAD+, with a maximum at about 300 nm and a tail extending to about 500 nm. Fig. 7 shows the absorbance at 300 nm as a function of the concentration of NAD+. The data lie on a theoretical curve calculated from the binding constant of NAD+ with Fragment A (8.3 μM). A molar absorbance coefficient of about 500 may be calculated from the data.

A similar change in absorbance and a quenching of fluorescence have been observed upon binding of NAD+ to glyceraldehyde phosphate dehydrogenase and certain other dehydrogenases. Both effects have been interpreted as resulting from a charge transfer complex between the nicotinamide moiety of NAD+ and a side chain of tryptophan in the protein. This interpretation is based largely on the work of Shifrin (20) on the model compound indolyldehydrogenase. The fluorescence of the indolyl ring is entirely quenched in the compound, and a long absorption tail (λmax 300 nm) is observed which is not present in either indole or nicotinamide alone. Presumably, this is also a valid model for the observed alterations of the optical properties of Fragment A. Michel et al. (19) have reported that Fragment A contains 2 tryptophan residues, while we have obtained a figure of 3 by various methods (21–24).

Mechanism of NAD+ Binding—Both the inactivation of EF-2 and the hydrolysis of NAD+ by Fragment A involve transfer of the ADPR moiety of NAD+ to an acceptor molecule (EF-2 or water, respectively). The possibility that a covalent ADPR-Fragment A intermediate might be involved in both reactions was considered and evidence for such an intermediate was sought.

1. Mixtures of [adenosine-14C]NAD+ (2.5 μM) and Fragment A (35 μM) in 50 mM Tris-HCl buffer, pH 8.2, were incubated for 2 min at 25° and chromatographed on columns of Sephadex G-25. The high and low molecular weight peaks were completely resolved, and there was no detectable radioactivity associated with Fragment A. The profile of radioactivity was superimposable with that of NAD+ chromatographed alone on the same column, indicating that binding of NAD+ to the fragment was rapidly reversible.

2. Mixtures of unlabeled NAD+ (1 mM) and Fragment A (100 μM) were incubated with [14C]nicotinamide (250 μM) in 50 mM sodium phosphate buffer, pH 7.8, at 37° for measurement of exchange of label into NAD+. Samples of 50 μl were removed at intervals of 20 min, and Fragment A was precipitated with 10% trichloroacetic acid. Samples of the supernatant fraction were then spotted on Whatman No. 3MM paper, NAD+ was separated from the nicotinamide by paper chromatography, and the radioactivity was determined. No evidence of exchange of nicotinamide with NAD+ was detected in any of the samples. Thus if an ADPR-Fragment A intermediate is formed, the nicotinamide moiety must remain firmly bound to the fragment.

3. Mixtures of [adenosine-14C]NAD+ and Fragment A were exposed to protein denaturants, followed by separation of the protein and ligand. Thus, for example, Fragment A (100 μM) was incubated with [14C]NAD+ (25 μM) at 25° for 5 or 30 min. After addition of SDS to 1%, the mixture was chromatographed on Sephadex G-25 equilibrated with 0.1% SDS. Again no radioactivity was found in the high molecular weight fraction. Similar results were obtained if guanidine HCl (3 M) was used in place of SDS or if electrophoresis on SDS-acrylamide gels was used instead of Sephadex for separation.

Thus no evidence for the postulated ADPR-Fragment A intermediate was obtained. NAD+ binding is rapidly reversible and presumably involves only noncovalent forces.
Binding of Adenine, Nicotinamide, and Related Compounds—
It has been reported that adenine, nicotinamide, and several
related compounds inhibit the ADP-ribosylation of EF-2 com-
petitively with respect to NAD+ (25). We have confirmed this
report and have compared the inhibitor constants of these com-
pounds with those obtained by measurement of competitive
binding to purified Fragment A (Table II). Both adenine and
nicotinamide bind competitively to Fragment A with \( K_i \) values
of about 30 and 220 \( \mu M \), respectively, as determined by dynamic
dialysis experiments (Fig. 8). Adenine is about an order of
magnitude more active as an inhibitor than adenosine and about
2 orders more active than AMP or ADPR. ADP and ATP are
also much weaker inhibitors than adenine as judged by fluores-
cence-quenching measurements. None of the adenine-containing
nucleotides quenches fluorescence, either alone or together
with nicotinamide, but the binding of adenine can be measured
by its reversal of the quenching produced by NAD+. Neither
adenine nor nicotinamide altered the absorbance at 360 nm (Fig. 7)
both although both could be shown to reverse the NAD+-induced absorbance by competitive
inhibition. The fact that nicotinamide fails to induce the 360-nm absorbance is expected inasmuch as its pyridine ring nitrogen is
uncharged, unlike that of NAD+, NMN, or N-methyl nicotin-
amide.

Binding of NADH, NADP+, and NAD+ Analogs—Both
dynamic dialysis and fluorescence-quenching methods were used
to study binding of pyridine nucleotides and analogs (Table III).
When the dialysis technique was employed, competition by un-
labeled analogs for binding of radioactive NAD+ was measured,
except in the case of NADH. The binding of labeled NADH
obtained by reduction of radioactive NAD+ was measured di-
nectly. Results from both dialysis and fluorescence measure-
ments showed that only thionicotinamid-A-D+ bound with an
affinity similar to that of NAD+ (\( K_i \approx 20 \mu M \)). Acetylpyridine-
AD+ (\( K_i = 440 \mu M \)) and deamino-NAD+ (\( K_i = 360 \mu M \)) showed
weak but significant binding. The affinities of NADP+,
\( \alpha \)-NAD+, and pyridinecarbaldehyde-AD+ were too low to measure.
NADH gave an apparent \( K_i \) of 140 \( \mu M \) by dialysis measure-
ments, but produced no significant quenching of fluorescence of
Fragment A.

Those compounds which failed to quench Fragment A fluores-
cence were also tested for capacity to reverse the NAD+-induced
quenching. Such reversal was never observed, indicating that
the absence of quenching was due to low affinity rather than low
efficiency of quenching by bound compound.

Substrate Activities of NAD+ Analogs—The capacities of vari-
ous NAD+ analogs to replace NAD+ as substrate in the ADP-
ribosylation of EF-2 were estimated. EF-2 (approximately
50 nm) was incubated at 25° in the presence of Fragment A for
periods up to 90 min with individual, unlabeled NAD+ analogs
(50 or 500 nm). Samples were removed at intervals and any
unreacted EF-2 was ADP-ribosylated during a 15-min incuba-
tion in the presence of [14C]NAD+ (400 nm) and additional Frag-

### Table II

Comparison of activities as inhibitors and relative affinities for
Fragment A of adenine- and nicotinamide-containing compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration required for half-maximal inhibition</th>
<th>Dissociation constant for Fragment A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[ADP-ribosylation of EF-2] ( \mu M )</td>
<td>[NAD+-glycohydrolyase] ( \mu M )</td>
</tr>
<tr>
<td>NAD+</td>
<td>30</td>
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<td>Adenosine</td>
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<tr>
<td>AMP</td>
<td>~3000</td>
<td>~3500</td>
</tr>
<tr>
<td>ADP, ATP</td>
<td>~3000</td>
<td>~6000</td>
</tr>
<tr>
<td>ADPR</td>
<td>~3000</td>
<td>~6000</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>~3000</td>
<td>2300</td>
</tr>
<tr>
<td>NMN</td>
<td>~3000</td>
<td>2300</td>
</tr>
</tbody>
</table>

* Measured from reversal of NAD+ quenching.
† No quenching or reversal of NAD+ quenching was observed at
concentrations up to 180 \( \mu M \).

### Table III

Comparison of substrate activity and affinity of various NAD+ analogs for Fragment A

<table>
<thead>
<tr>
<th>Analog</th>
<th>Relative substrate activity(^a)</th>
<th>Dissociation constant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[Dynamic dialysis] ( \mu M )</td>
</tr>
<tr>
<td>NAD+</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Thionicotinamide-AD+</td>
<td>90</td>
<td>15</td>
</tr>
<tr>
<td>Deamino-NAD+</td>
<td>7</td>
<td>440</td>
</tr>
<tr>
<td>Acetylpyridine-AD+</td>
<td>11</td>
<td>362</td>
</tr>
<tr>
<td>Pyridinecarbaldehyde-AD+</td>
<td>0</td>
<td>3100</td>
</tr>
<tr>
<td>Acetylhypdrideaminoad+</td>
<td>1</td>
<td>3200</td>
</tr>
<tr>
<td>NADP+</td>
<td>100</td>
<td>3200</td>
</tr>
<tr>
<td>NADH</td>
<td>0</td>
<td>140</td>
</tr>
</tbody>
</table>

* Expressed as per cent of NAD+ activity in the assay (calcu-
lated from data in Fig. 9).
† No significant quenching observed (<10% at 250 \( \mu M \)).
\( ^b \) Not measured.

---

**Fig. 8.** Measurements of binding of adenine and nicotinamide to Fragment A by dynamic dialysis. Experiments were performed as described in Fig. 4, except that a single addition of unlabeled NAD+ analog was made 5 min prior to titration with unlabeled NAD+. NAD+ (••••••••); adenine, 90 \( \mu M \) (○○○○○○○○); nicotinamide, 280 \( \mu M \) (△△△△△△△).
Fig. 9. Substrate activity of NAD+ analogs. Nonradioactive NAD+ analogs (50 or 500 nM) were incubated in the standard ADP-ribosylation reaction mixture. All the components except Fragment A were mixed at 0° and after initiation of the reaction by the addition of Fragment A, the mixtures were incubated at 25° for periods up to 90 min. Samples of 250 μl were withdrawn at the times indicated and any unreacted EF-2 was ADP-ribosylated during an additional 15-min incubation in the presence of (adenosine-3',5')cyclic NAD+ (400 nM) and additional Fragment A (800 nM). The reaction was terminated by addition of 500 μl of 10% trichloroacetic acid. The precipitates were collected on Whatman glass fiber discs, washed with 5% trichloroacetic acid, and dried; the radioactivity was counted in a Nuclear Chicago low background counter. NAD+, 50 nM (□); thiocticominamide-AD+, 50 nM (○); acetylpyridine-AD+, 50 nM (△); deamin-NAD+, 50 nM (●); acetylpyridinedeamino-AD+, 500 nM (△); pyridinecarbaldehyde-AD+, pyridinecarbaldehyde-deamin-NAD+, 500 nM; a-NAD+, NADP+, NADPH, 50 nM (−−−). Substrate activity of NADH was measured after reduction of the radioactive oxidized nucleotide (0.5 μM) for 30 min at 25°. Radioactive NADH (50 nM) was assayed in the standard reaction mixture. The presence of ethanol or alcohol dehydrogenase alone had no effect on the inactivation of EF-2.

The extent of incorporation of label permitted estimation of the substrate activity of the various analogs by difference (Fig. 9). The plateau in the minimal level of incorporation may have resulted from the presence of an excess of EF-2 over pyridine nucleotide during the initial incubation or partial reversal of the reaction during the second incubation.

The results correlate well with the data from binding experiments. Thiocticominamide-AD+ was the only analog with substrate activity similar to that of NAD+. In agreement with results reported by Hunko et al. (3) and Goor and Rappehohmer (26), both acetylpyridine-AD+ and deamin-NAD+ reacted at rates of about 11 and 7%, respectively, of that of NAD+. NADH was measured after reduction of the radioactive oxidized nucleotide (0.5 μM) for 30 min at 25°. The activity of NADH in this system is in reasonable correlation with the measured affinity of NADH for Fragment A. The activity of NADH in this system is in reasonable correlation with the measured affinity of NADH for Fragment A, especially if one considers close to that previously reported, at least in the system we employ.

The activity of NADH in this system is in reasonable correlation with the measured affinity of NADH for Fragment A, especially if one considers the possible exchange of labeled NAD+ with unlabeled competitor NADH. The latter possibility would not seem unlikely in view of the impurity of the EF-2 we have used, and it may in part also explain the stronger inhibition observed in the rat liver extract (25). In addition we have found that NADH competes only weakly, if at all, with NAD+ in the NAD+-glycohydrolase reaction catalyzed by Fragment A. Inhibition of this reaction was negligible at concentrations of 10 μM or less, and half-maximal inhibition required at least 250 μM NADH.

Protection of Fragment A against Proteolysis—When Fragment A was incubated with moderate concentrations of either trypsin or chymotrypsin, it was slowly inactivated, and there were concomitant declines in the capacity to bind NAD+ and the intensity of fluorescence at 333 nm (Fig. 11). In the completely digested fragment the quantum yield was about 30% of its original value, and the emission maximum had shifted from 333 to 355 nm, the latter being the maximum of free tryptophan in solution.

NAD+ at saturating concentrations (1 μM) protected the fragment completely from chymotrypsin action for at least 60 min and almost completely from trypsin action. Adenine provided partial protection from both proteases. The protective capacities of various NAD+ analogs correlated with their intrinsic affinities for the fragment.
Degradation of Fragment A in the presence and absence of protective compounds was monitored by SDS gel electrophoresis. Loss of enzymic activity was accompanied by a decline in the intensity of the Fragment A band, and no bands representing intermediates in the degradation process were detected during tryptic or chymotryptic digestion in the presence or absence of protective ligands.

Conformational Probes—Two techniques were employed to detect possible major changes in conformation upon binding of NAD\(^+\) to Fragment A.

1. Fragment A containing approximately two dansyl groups per molecule was used for measurements of fluorescence polarization. The dansyl groups did not reduce the capacity of the fragment to bind NAD\(^+\) or its enzymic activity. The rotational relaxation time of conjugated Fragment A was determined at pH 8.0 from a plot of 1/\(P\) against \(T/\eta\). A linear dependence was observed between 8 and 35\(^{\circ}\), and no difference was observed in the curves obtained in the presence and absence of NAD\(^+\) (Fig. 12). The value calculated for the rotational relaxation time of the fragment was 17\(\,\text{ns}\). The ratio of this value to that predicted for a sphere of molecular weight 24,000 is 1.09. Thus the polarization of the dansylated groups may therefore be used as a valid indicator of changes in the rotational relaxation time of the fragment.

2. Measurement of the sedimentation coefficient of Fragment A by the method of differential sedimentation (17) yielded a value of 2.19\(\,\text{S}\) using ribonuclease as a standard (1.84\,S). We should have been able to detect differences as small as 0.016\,S with this technique. No differences in the sedimentation coefficient between free and NAD\(^+\)-complexed Fragment A was observed.

These tests revealed no gross alteration in the native conformation of Fragment A upon the binding of NAD\(^+\).

**DISCUSSION**

From the results presented, it is clear that Fragment A contains a single NAD\(^+\) binding site with a \(K_d\) of about 8\,\mu M. The site interacts with both the adenine and nicotinamide moieties of NAD\(^+\) as evidenced by competitive binding experiments and in particular, in the case of the nicotinamide portion, by optical evidence (induction of absorbance at 360\,nm, and quenching of protein fluorescence). The binding of NAD\(^+\) did not produce a gross alteration of conformation of Fragment A detectable by either fluorescence polarization or differential sedimentation, although it did significantly increase the resistance to trypsin or chymotrypsin.

The properties of the NAD\(^+\) binding site seem consistent with the idea that it is active in catalysis of both the NAD\(^+\)-glycohydrolase and the NAD\(^+\).EF-2-ADPR-transferase reactions. In the case of the NAD\(^+\)-glycohydrolase reaction, this relationship is apparent inasmuch as it has been shown unequivocally that Fragment A itself, and not a contaminant, is responsible for this low activity (Fig. 3). There are further correlations by virtue of the similarities of the \(K_i\) values of various competitive inhibitors of the reaction to those for the binding of NAD\(^+\) (Table II) and by the data in Fig. 13, which show that the \(K_m\) for NAD\(^+\) is close to 8\,\mu M.

In the case of the NAD\(^+\).EF-2-ADPR-transferase activity, it is not evident a priori that Fragment A must contain an intact NAD\(^+\) binding site which is active in this reaction. Thus one could conceive that the site might lie entirely on EF-2 or at the interface of a Fragment A-EF-2 complex, and in fact the latter...
The possibility has been proposed in an earlier model (25). However, the evidence presented here leaves little doubt that the NAD$^+$ binding site on Fragment A is that which is involved in transfer of ADPR to EF-2. (a) The measured $K_d$ for NAD$^+$ with Fragment A (5 μM) correlates well with published values of the $K_m$ of NAD$^+$ for ADPR transfer (5 μM) (3, 25). (b) The patterns of specificity and affinity for both inhibitors and substrates correlate well between the reaction and NAD$^+$ binding to Fragment A (Tables II and III). (c) Simply the existence of the NAD$^+$-glycohydrolylase activity is the most convincing evidence, however. Thus when NAD$^+$ is bound to its cognate site on Fragment A, the nicotinamide-ribose linkage is accessible to nucleophilic substitution by water. It seems probable, therefore, that it might also be highly reactive with an appropriately juxtaposed nucleophilic group of EF-2. Although the nature of the single acceptor site on EF-2 is not known, there is evidence from two laboratories now that a basic residue is involved (26, 27).

On the basis of this evidence, it seems clear that NAD$^+$ binds initially to Fragment A during transfer of ADPR to EF-2. Inasmuch as the binding is apparently noncovalent and rapidly reversible, the reaction presumably proceeds through a ternary intermediate containing NAD$^+$ and EF-2 bound to adjacent sites on Fragment A. EF-2 presumably binds such that its ADPR acceptor site is contiguous with the reactive nicotinamide-ribose linkage of NAD$^+$, and following transfer of the ADPR intermediate containing NAD$^+$ and EF-2 bound to adjacent sites on Fragment A, the complex dissociates into ADPR-EF-2, nicotinamide, H$^+$, and Fragment A.

An earlier model based largely on kinetic measurements (25) was similar in that the reaction was concerted and proceeded through a ternary complex. However, a different mode of binding of NAD$^+$ within the ternary complex was proposed. Thus the adenine portion was supposed to bind to the catalytic protein (thought to be toxin at the time this work was done) whereas the nicotinamide moiety bound to EF-2. The evidence for this was not compelling, however, and was obtained in an impure system and before the reaction was well characterized. Thus we feel that there is a firmer basis for the present model.

There are two reports of binding of NAD$^+$ to toxin, based on studies which were performed before the existence and significance of Fragment A were realized (28, 29). Our own preliminary studies with whole toxin indicate that it binds far less than a molar equivalent of NAD$^+$, however, and we are not yet confident that whole toxin binds NAD$^+$ at all if all contaminating fragments are rigorously removed. A future report will characterize the interaction of intact and nicked toxin with NAD$^+$ and related compounds.

Other instances are known in which transferases catalyze hydrolysis of a substrate which is normally involved in a group transfer reaction. The best known example of this is hexokinase in which ATP is hydrolyzed to ADP and P$\text{i}$ in the absence of glucose at a rate above 5 $\times$ 10$^{-6}$ the rate of the reaction in the presence of glucose (30). Many explanations are possible for the large differences in rates in the presence and absence of the normal acceptor. Meaningful speculation on this question in relation to the activity of Fragment A must await identification of the group on EF-2 to which ADPR becomes attached and other information about the interaction of EF-2 with Fragment A.

The rate of hydrolysis of NAD$^+$ by Fragment A has been estimated to be less than 2 $\times$ 10$^{-6}$ that of the transfer of ADPR to EF-2 under conditions of saturation with NAD$^+$. It can be calculated from the turnover number (0.05 mole of NAD$^+$ hydrolyzed per min per mole of Fragment A) and the concentration of NAD$^+$ (0.5 mm) and Fragment A (10$^{-3}$ m or less) in toxin-treated cells, that an entirely negligible fraction of the NAD$^+$ should be hydrolyzed per hour (less than 10$^{-9}$). Thus it seems unlikely that the NAD$^+$-glycohydrolylase activity plays a significant role in the toxicity of diphtheria toxin. This conclusion is consistent with the fact that glycolysis and respiration both continue normally in cultured cells long after protein synthesis has ceased (31).

Note Added in Proof—Reference was inadvertently omitted in the discussion to the report of Hayes and Kaplan (11) on the interaction of NAD$^+$ with an enzymically active fragment of toxin. As detected by nuclear magnetic resonance spectroscopy, binding of NAD$^+$ caused perturbation of the environment of nicotinamide ring protons, and there was further indication that the interaction involved an aromatic region of the protein. In addition, we have learned (M. B. Hayes, personal communication) that these workers also independently observed NAD$^+$ glycohydrolylase activity of the fragment.

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Interaction of Fragment A from Diphtheria Toxin with Nicotinamide Adenine Dinucleotide

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