The Diphtheria Toxin-dependent Adenosine Diphosphate Ribosylation of Rat Liver Aminoacyl Transferase II

GENERAL CHARACTERISTICS AND MECHANISM OF THE REACTION*

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RONALD S. GOOR AND ELIZABETH S. MAXWELL

From the Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Diphtheria toxin is known to catalyze a reversible reaction in which the adenosine diphosphate ribose moiety of NAD is transferred to aminoacyl transferase II, resulting in the concomitant loss of transferase activity in protein synthesis. Some general characteristics of the reaction are reported and a mechanism is proposed.

The rate of the reaction is proportional to toxin, aminoacyl transferase II, and NAD concentrations, but, in the presence of excess NAD, the extent of the reaction is dependent only on the amount of aminoacyl transferase II present. ADP ribosylation occurs at all temperatures between 0-40° with a maximum at 20°. The adenosine diphosphoribosylaminoacyl transferase II bond is stable to heating at 90° for 15 min in 5% trichloracetic acid.

The following random mechanism for the reaction is proposed on the basis of results from kinetic studies, use of inhibitors of the reaction and isotope exchange reactions, as well as our failure to find a partial reaction. A ternary intermediate composed of toxin, NAD, and aminoacyl transferase II is formed by a series of different binary reactions. The results support the participation of all combinations of binary reactions in the formation of the ternary complex. Toxin is bound to the adenosyl moiety of NAD, as well as to the transfer enzyme. Aminoacyl transferase II is bound to the NMN moiety of NAD. The proposed ternary intermediate consisting of toxin, aminoacyl transferase II, and NAD breaks down rapidly to the final products, adenosine diphosphoribosylaminoacyl transferase II, nicotinamide, and toxin.

Diphtheria toxin inhibits the incorporation of amino acids into protein by mammalian cells (1) and by systems in vitro derived from these cells (2). NAD is required specifically for this inhibition in vitro (2, 3). The site of action of toxin and NAD is aminoacyl transferase II (4), a soluble enzyme required together with aminoacyl transferase I and ribosomes for transfer of amino acids from aminoacyl transfer RNA into protein.

Transferase II has been shown (5-8) to function in polypeptide chain extension by catalyzing translocation of the nascent chain from the unreactive acceptor (aminoacyl) site on the ribosome to the donor (peptidyl) site in which the chain can again react with an incoming aminoacyl-tRNA or with puromycin to form a peptide bond (9-11). Translocase activity of transferase II, independent of transferase I and aminoacyl-tRNA, is measured by a GTP-dependent stimulation of the number of polypeptide chains that can react with puromycin (5). Toxin and NAD inhibit the stimulation of the puromycin reaction by transferase II (5). Like the analogous G factor in Escherichia coli, transferase II also exhibits a ribosome-dependent GTPase activity (6, 12). Toxin and NAD inhibit this activity (6). Common inhibition by toxin and NAD of transferase II-dependent GTPase activity, translocase activity, and amino acid incorporation has led to the conclusion that these activities are catalyzed by a single protein in partially purified transferase II or that they share a requirement for such a protein.

The inactivation of transferase II can be reversed by toxin and high concentrations of nicotinamide (13-15) and recent results indicate that toxin exerts its effect by catalyzing the following reversible reaction (14, 15).

\[ \text{NAD}^+ + \text{Transferase II} \rightleftharpoons \text{ADP-ribose} - \text{Transferase II} \]

(active) \quad (inactive)

\[ + \text{nicotinamide} + \text{II}^+ \]

The ADP ribosylation of transferase II intimately parallels inactivation of transferase II in amino acid-incorporating systems (15) and reversal of ADP ribosylation by nicotinamide results in restoration of transferase II activity (15).

In the present report, we have investigated some general characteristics of the reaction and have pursued and extended our understanding of the mechanism of ADP ribosylation.

1 The abbreviations used are: transferases I and II, aminoacyl transferases I and II; ADP-ribose, adenosine diphosphate ribose; ADP-ribose-transferase II, adenosine diphosphoribosyl-aminoacyl transferase II.
of ADP ribosylation of transferase II by toxin and NAD is a modification of the method described by Honjo et al. (15). Unless otherwise specified, each reaction mixture (0.2 ml) contained 1 μmole of Tris-HCl buffer, pH 7.3, 0.5 μmole of KCl, 20 μmole s of dithiothreitol, 0.1 μmole of MgCl₂, 0.15 μg of diphtheria toxin, 250 μg of transferase II, and 150 to 200 μmole s of 3H-NAD labeled in the adenine moiety (3.6 \times 10^6 cpm). Length and temperature of incubation are indicated in each experiment. The reaction was stopped by adding 1 μg of carrier bovine serum albumin and 2 ml 5% trichloracetic acid. The precipitate was washed twice by centrifugation with 5% trichloracetic acid, collected on Millipore filters, washed again, dried, and counted in a toluene-base solution (Liquifluor, Pilot Chemicals, Inc., Watertown, Massachusetts) in a Nuclear-Chicago Mark I scintillation counter. In some of the later experiments a method that yielded higher counting efficiency was used. The washed trichloracetic acid precipitates were plated on glass filters (Whatman, GF/C), dried with two aliquots of 5 ml of ether, transferred to vials, and the precipitates dissolved with 0.2 ml of Nuclear-Chicago solubilizer, and counted in 5 ml of Liquifluor; before counting, the scintillation fluid was neutralized with 0.01 ml of 12 N H₂SO₄. This treatment increased the efficiency to 30%.

Amino Acid Incorporation in Cell-free System—Poly rU-directed incorporation of 14C-phenylalanine into trichloracetic acid-precipitable material was carried out in the following system. Each reaction mixture contained 3 μmole s of MgCl₂, 40 μmole s of mercaptoethanol, 25 μmole s of Tris-HCl buffer, pH 7.3, 0.188 μmole s of GTP, 100 μg of poly rU, 100 to 200 μg of ribosomes, 100 μg of transferase II, 200 μg of transferase I, and 13.6 μg of 14C-phenylalanyl-tRNA in a final volume of 0.5 ml. Incubation was for 10 min at 37°C. The reaction was stopped by precipitating with 5 ml of 5% trichloracetic acid. The precipitate was washed and filtered onto Millipore filters and counted in Liquifluor by liquid scintillation counting.

Paper Chromatography—Reaction mixtures were either applied directly to paper (Schleicher and Schuell No. 589 Orange ribbon) or several successive aliquots or were first precipitated with 67% ethanol (final concentration), the precipitate removed, and the supernatant evaporated to a volume convenient for spotting onto the paper. In those experiments, where 3H-NAD labeled in the nicotinamide moiety was used, the paper was developed for 4 hours by descending chromatography with Solvent C of Pries and Handler (21) modified slightly to contain 7 parts of 95% ethanol to 3 parts of 1 N ammonium acetate buffer, pH 4.8. NAD and nicotinamide standards were cochromatographed with the reaction mixtures. Rₚ values in this system are NAD, 0.19, and nicotinamide, 0.81. In those experiments where 3H-NAD labeled in the adenine moiety was used, the paper was developed for about 24 hours. NAD, ADP-ribose, and AMP standards were cochromatographed with the reaction mixtures. The appropriate spots were located by quenching of ultraviolet light, cut out, eluted with 1 ml of water, and counted in 10 ml of Bray's (dioxane-base) by liquid scintillation. Recovery of radioactivity was better than 70%.

RESULTS

Characteristics of ADP Ribosylation Reaction

Honjo et al. (15) reported, and we have confirmed, that toxin is specifically required to catalyze the ADP ribosylation of transferase II. Toxoid, even at a level of 12 μg, has no activity in the
The higher level of incorporation observed in the reaction performed in potassium phosphate buffer, pH 7.6, containing 0.15 mM of toxin and 50 mM dithiothreitol and incubated for 10 min at the specified temperature. Points on the lower curve were obtained from an identical reaction performed in 0.01 M Tris-HCl, pH 7.3 (at room temperature), containing 0.15 mM of toxin but no dithiothreitol. In order to measure reversal, ADP-ribose-transferase II was prepared by incubating transferase II, 1H-NAD labeled in the adenine moiety (0.45 mmole) and toxin together for 1 hour at 0°. The mixture was then treated with an excess of streptococcal NADase (4500 units) for 1 hour at 0°. Aliquots of this mixture were then treated with 6 mmole of nicotinamide and incubated for 10 min at the specified temperatures before stopping the reaction with trichloroacetic acid. The three curves represent reactions performed under identical conditions, but with the following differences: 0.05 mM acetate buffer, pH 5.6 (top curve); 0.05 mM potassium phosphate buffer, pH 7.6 (middle curve); 0.01 M Tris-HCl buffer, pH 7.3, at room temperature (bottom curve). The results are expressed as the tritium (counts per min) released from the starting material after incubation at each temperature.

The initial rate of ADP ribosylation of transferase II is a linear function of the amount of toxin up to 0.4 mM of the NAD concentration to 4.8 µM, and of the transferase II concentration to 1.2 mg per ml. We have confirmed the finding of Honjo et al. (15) that, in the presence of excess NAD, the extent of the reaction is constant. The turnover number of toxin under conditions of the assay (low NAD concentration) is quite low but it can be increased if the NAD concentration is raised. That toxin is catalytic, however, is indicated by the fact that 0.01 µmole of toxin carried out the incorporation of 10 µmoles of ADP-ribose into ADP-ribose-transferase II from NAD.

The ADP-ribose-transferase II bond was found to be stable when the trichloroacetic acid precipitate was heated for as long as 15 min at 90°.

The incorporation of labeled ADP-ribose of a known specific activity into the transferase II fraction allows one to calculate the purity of the transferase II. The incorporation of ADP-ribose into ADP-ribose-transferase II from Tris buffer at low temperatures has been used in a later section to study the forward reaction under conditions where reversal does not proceed.

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The incorporation of labeled ADP-ribose of a known specific activity into the transferase II fraction allows one to calculate the purity of the transferase II. Assuming that only 1 ADP-ribose residue is incorporated per molecule of transferase II, we calculate that the transferase II used in these experiments is only about 1% pure, a degree of purity of the same order as that used by Honjo et al. (15).

**Mechanism of Reaction**

**Inhibitors of ADP Ribosylation of Transferase II**—Inhibitors in the first group are competitive with respect to both transferase II and NAD and include GMP, GDP, GTP, β,γ-GTP-methylene diphosphonate, GTP, and β,γ-GTP-methylene diphosphonate. GTP and β,γ-GTP-methylene diphosphonate were examined because they are known to interact with transferase II (6) and to influence its association with ribosomes (7, 8).

Fig. 2A shows the rate of incorporation of 1H-ADP-ribose into trichloroacetic acid-precipitable material in the absence of GTP, in the presence of 7.5 mM GTP, and in the presence of GTP and ribosomes. Fig. 2B shows the same experiment with β,γ-GTP-methylene diphosphonate replacing GTP. It is clear that both

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*Y. Nishizuka, personal communication.*
GTP and the analogue inhibit the ADP ribosylation of transferase II and that ribosomes enhance this inhibition. GTP and $\beta,\gamma$-GTP-methylene diphosphonate inhibit the initial rate rather than the extent of the ADP ribosylation of transferase II. ITP can replace GTP as an inhibitor and is approximately as active. GMP and GDP inhibit the ADP ribosylation of transferase II by competing with NAD and, but are less effective than GTP on a molar basis. The order of inhibitory activity is $\beta,\gamma$ GTP methylenthylenediphosphonate > GTP = ITP > GDP > GMP.

Inhibition of the initial rate of ADP ribosylation of transferase II by two different concentrations of GTP as a function of either the NAD or the transferase II concentration is shown by the Lineweaver-Burk reciprocal plots in Fig. 3. The inhibition is competitive with respect to both NAD and transferase II. The apparent $K_m$ for NAD is 5 $\mu$M and the $K_i$ for GTP is 4.9 $\mu$M. A value for the $K_m$ of transferase II is not reported because of the impurity of the transferase II preparation. GTP (7.5 mM) also inhibits the re-formation of NAD from ADP-ribose-transferase II and nicotinamide by toxin.

Nicotinamide also inhibits the ADP ribosylation of transferase II and is competitive with both NAD and transferase II. The $K_i$ for NMN is 3.3 mM. NMN does not inhibit the reverse reaction, the re-formation of NAD from ADP-ribose-transferase II and nicotinamide by toxin.

Since NAD is known to bind to toxin and AMP constitutes half of the NAD molecule, it seems likely that members of the A series inhibit the ADP ribosylation of transferase II by competing with NAD for its binding site on toxin. Supporting evidence for such a mechanism was provided by Sperti and Montanaro (23) who showed by equilibrium dialysis and quenching of protein fluorescence that adenine competes with NAD for the same binding site on toxin and that the dissociation constants for NAD and for adenine are of the same order (10$^{-4}$ M). Members of the A series inhibit the reaction in the following order of effectiveness: adenine > adenosine > AMP > ADP > ATP. Fig. 4 shows the relative effectiveness of various concentrations of adenine, adenosine, NMN, and GTP in inhibiting ADP ribosylation of transferase II. A comparison of the concentrations needed for 50% inhibition shows that adenine is an order of magnitude more active than adenosine or NMN. Adenosine and NMN are about 34 times more active on a molar basis than GTP. Experiments not shown here indicated that adenosine is at least an order of magnitude more active than AMP and ADP or ATP. Adenine and adenosine do not cause an apparent inhibition by exchanging with the labeled adenine in the $^3$H-NAD and thereby diluting the label. This was determined by incubating $^3$H-NAD, toxin, transferase II, and adenine (or adenosine) and then chromatographing the mixture as described under "Materials and Methods." None of the radioactivity appeared in either the adenine or adenosine spots.

The most active member of the series, adenine, was used for the kinetic studies shown in Fig. 5. Adenine is competitive with respect to NAD and noncompetitive with respect to transferase II. The $K_i$ for adenine is 36 $\mu$M. Supporting evidence is provided by Sperti and Montanaro (23) who report $K_i$ for adenine as 38.5 $\mu$M from equilibrium dialysis experiments performed with toxin, NAD, and adenine in the absence of transferase II.

Using equilibrium dialysis, we have confirmed the finding of Sperti and Montanaro (23) that nicotinamide does not bind to toxin to an appreciable extent.

Inhibitors in the second group, which includes all members of the adenine-ATP (A) series and NADH, are competitive with respect to NAD and noncompetitive with respect to transferase II.
fluorescence that 2 moles of NADH bind per mole of toxin and that the affinity of toxin is higher for NADH than for NAD. Nonetheless, Goor and Pappenheimer (3) demonstrated that NADH is incapable of replacing NAD in the inhibition by toxin of amino acid incorporation in cell-free systems. These observations lead to the prediction that NADH should be a very potent inhibitor of the ADP ribosylation of transferase II by toxin and NAD. This prediction is confirmed. NADH is competitive with respect to NAD and noncompetitive with respect to transferase II. The $K_i$ for NADH is 0.23 $\mu$m; it is approximately two orders of magnitude more active than adenine on a molar basis and is the most active inhibitor tested. NADH (0.55 $\mu$m) also inhibits the reverse reaction.

The pyrimidines, cytosine, CMP, CDP, uridine, UMP, and UDP did not inhibit the forward reaction at all at a concentration of 7.5 $\mu$m. CTP and UTP showed slight inhibitory activity at this concentration consistent with their binding MgCl$_2$ in the reaction mixture. The concentration of MgCl$_2$ normally present in the reaction mixture caused a slight stimulation of the initial rate of ADP ribosylation.

Table I provides a summary of the studies with inhibitors of the ADP ribosylation of transferase II by toxin and NAD.

**Evidence for Concerted Reaction**—Earlier results (3, 23, 24) have shown that toxin binds NAD. If this represents the first step in the reaction, then the succeeding reaction may follow either of two different mechanisms. In one case the mechanism may be concerted, i.e. the toxin-NAD complex will not react until transferase II is added and only the toxin-NAD-transferase II complex is active in splitting NAD to give the reaction products, nicotinamide and ADP-ribose-transferase II.

**Model A**

1. Toxin + NAD $\rightarrow$ toxin-NAD

2. Toxin-NAD + transferase II $\rightarrow$ toxin-NAD-transferase II

3. Toxin-NAD-transferase II $\rightarrow$ toxin

   + ADP-ribose-transferase II + nicotinamide

The toxin-NAD-transferase II complex may be very unstable and thus very short-lived. On the other hand, the reaction may follow the course given below.

**Model B**

1. Toxin + NAD $\rightarrow$ toxin-NAD

2. Toxin-NAD $\rightarrow$ ADP-ribose-toxin + nicotinamide

3. ADP-ribose-toxin + transferase II $\rightarrow$

   ADP-ribose-transferase II + toxin

In this case, a half reaction in which NAD is split and free nicotinamide released should be detectable and the intermediate ADP-ribose-toxin should be identifiable and active in transferring the ADP-ribose residue to transferase II in the absence of free NAD.

Toxin in a large molar excess relative to NAD slowly cleaves the NAD in the absence of transferase II, releasing nicotinamide which can be identified by paper chromatography. Dithiothreitol fails to stimulate the rate of this cleavage even at a concentration that stimulates ADP ribosylation. Both adenine and nicotinamide inhibit the cleavage. If this cleavage is part of the reaction mechanism, then ADP-ribose-toxin should be formed concomitant with the release of nicotinamide. No evidence for an ADP-ribose-toxin intermediate could be found. Thus, $^3$H-NAD labeled in the adenine moiety was incubated with toxin under conditions leading to cleavage and the reaction mixture then passed through a Sephadex G-50 column. No tritium eluted in the excluded volume with the proteins and all the label was found in the included volume. These results suggest that the $^3$H-ADP-ribose that is split from NAD by toxin is probably released and that the cleavage is probably hydrolytic. It may be either a side reaction of toxin carried out at a very slow rate or a reaction catalyzed by a contaminant NADase present in the toxin preparation in extremely low concentrations. Recent results support the latter possibility. As toxin was purified to homogeneity, the hydrolytic cleavage of NAD in the absence of transferase II decreased to zero and the ratio of trimethylammonium precipitable ADP-ribose-transferase II formed in the presence of transferase II to nicotinamide released approached one.

Addition of transferase II to the reaction mixture causes the formation of $^3$H-ADP-ribose-transferase II which is excluded from Sephadex G-50. Subsequent incubation of the $^3$H-ADP-ribose-transferase II with nicotinamide leads to the re-formation of $^3$H NAD, identified by paper chromatography.

The above results suggest that the reaction mechanism is concerted and that the reactive intermediate is a ternary complex.

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**Table I**

| Inhibitor | Group | Inhibition of | Forward $K_i$ |
|-----------|-------|---------------|--------------|
|           |       | Reverse reaction | mM          |
| GTP (G series) | 1 | Yes | Yes | 4.8 |
| NMN | 1 | Yes | No | 3.3 |
| Nicotinamide | 1 | Yes | Substrate | 0.21 |
| Adenine (A series) | 2 | Yes | ? | 0.056 |
| NADH | 2 | Yes | Yes | 0.00023 |
predicts exchange only in the presence of transferase II. The presence of added transferase II is probably the result of a small amount of unreacted transferase II remaining after formation of [1H]-ADP-ribose-transferase II.

Results of isotope exchange reactions confirm the existence of a ternary intermediate (Model A) and rule out the possibility of a partial reaction involving ADP-ribose-toxin (Model B). If the reaction occurred by a ping-pong mechanism (Model B), isotope exchange would be expected between [1H]-nicotinamide and NAD upon incubation with toxin in the absence of transferase II. This was never observed. On the contrary, evidence was obtained for a reaction mechanism of the Model A type by the following isotope exchange experiment. [1H]-ADP-ribose-transferase II labeled in the adenine moiety was incubated with toxin and NAD in the presence and absence of transferase II. According to Model B, exchange of tritium into NAD would occur when transferase II was present. Model A, however, predicts exchange only in the presence of transferase II. The results in Table II show the exchange of tritium into NAD occurred only when transferase II was added to the incubation mixture. The low level of radioactive NAD formed in the absence of added transferase II is probably the result of a small

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**Table II**

| Transferase II | 1H-NAD |
|---------------|--------|
| Absent        | 157    |
| Present       | 662    |

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Inhibition of the initial rate of ADP ribosylation by GTP and \( \beta,\gamma\)-GTP-methylene diphosphonate is enhanced by ribosomes (Fig. 2, A and B). Skogerson and Moldave (7, 8) showed that transferase II binds to ribosomes only in the presence of GTP and Raeburn et al. (6) found that GTP binds to partially purified transferase II. \( \beta,\gamma\)-GTP-Methylene diphosphonate allows transferase II to bind to ribosomes (8) and it probably can replace GTP in binding to transferase II. As already discussed, the inhibition of ADP ribosylation by GTP and \( \beta,\gamma\)-GTP-methylene diphosphonate probably results from their binding to transferase II. Since the binding of GTP to transferase II is reversible while the ADP ribosylation is irreversible under the conditions of the experiment, inhibition of ADP ribosylation by GTP (\( \beta,\gamma\)-GTP-methylene diphosphonate) is expected to affect only the initial rate and not the extent of the reaction. The addition of ribosomes to the reaction changes the situation considerably. Goor and Pappenheimer (19) and Gill et al. (14) showed that ribosomes compete with the toxin-NAD complex for transferase II, which is protected from toxic inactivation when bound to ribosomes. When ribosomes are included in the reaction mixture with GTP, transferase II, toxin, and NAD, the fraction of transferase II that is protected from ADP ribosylation by toxin increases since a certain percentage of the transferase II is now bound to ribosomes where toxin cannot inactivate it.

However, transferase II splits GTP in the presence of ribosomes (6, 12) and may turn over, i.e., re-enter the pool of free transferase II where it is once more susceptible to toxin. Thus, even in the presence of ribosomes and GTP, all of the transferase II added to the reaction eventually will be ADP ribosylated. The GTP analogue \( \beta,\gamma\)-GTP-methylene diphosphonate, however, is incapable of being split by transferase II on the ribosomes, and
Toxin also binds NADH and, in fact, exhibits a higher affinity for NADH than for NAD (24). This is consistent with the observation that NADH has a lower $K_d$ than adenine (see Table I). Alternatively, the random mechanism predicts that toxin can bind transferase II as a first step (Step Ib). Until homogeneous transferase II and toxin are available detection and measurement of this binding remains extremely difficult and thus we have not yet attempted to find it.

A third possible binary reaction involving the binding of the two substrate molecules NAD and transferase II is pictured in Step Ic. Preliminary evidence (6) indicates that NAD does bind to partially purified transferase II.

The toxin-NAD complex formed in Step Ia is pictured in Step IIa as binding to transferase II via the nicotinamide-ribose moiety to form a ternary complex of toxin, NAD, and transferase II. This would explain the kinetics of inhibition by NMN and nicotinamide which are competitive with both NAD and transferase II and are pictured as inhibiting Steps Ic, IIa, and IIb. With the use of equilibrium dialysis, we confirmed the finding of Sperti and Montanaro (23) that nicotinamide does not bind to toxin. Furthermore, nicotinamide does not inhibit the binding of NAD to toxin (23). We thus propose that it binds to transferase II in the site of ADP ribosylation, thereby preventing the entry of both NAD and the toxin-NAD complex. NMN may act in a manner similar to nicotinamide. Alternatively, the ternary complex may be formed by the binding of NAD to the toxin-transferase II complex (Step IIb). In this case, NAD binding to the toxin-transferase II complex could be inhibited by both adenine and NADH as well as by NMN and nicotinamide. According to the model for the random mechanism, binding of NAD to toxin is unaffected by the prior binding of transferase II to toxin. Thus, NAD binds to toxin with the same association constant in Steps Ia and IIb; this is consistent with the observation that inhibition by adenine and NADH is competitive with NAD and noncompetitive with transferase II.

The transferase II-NAD complex pictured in Step Ic may bind to toxin to produce the ternary complex once again (Step IIc). Adenine and NADH would be expected to inhibit this step.

Since GTP is known to bind to transferase II (6) and since it inhibits competitively with respect to both NAD and transferase II, the guanosine nucleotides may inhibit by binding to transferase II either in or near the site of ADP ribosylation and competing with the entry of either NAD or the toxin-NAD complex or, alternatively, by binding at a distant site and causing a conformational change in transferase II which lowers its affinity for NAD and the toxin-NAD complex.

The results of kinetic experiments shown in Fig. 6 and of the isotope exchange reactions predict the existence of a ternary intermediate consisting of toxin, NAD, and transferase II (Steps IIa to $c$). Goo and colleagues (13) proposed such a complex as the final inactive form of transferase II before toxin was shown to be an enzyme. Such a complex would have been stable. However, neither Honjo et al. (15) nor Gill et al. (14) were able to find the complex by the use of $^{32}P$-labeled toxin, and Honjo et al. (15) actually separated toxin from the inactivated transferase II (ADP-ribose-transferase II) on a hydroxylapatite column.
dave, using ultracentrifugal techniques, have obtained direct evidence for the proposed ternary intermediate.3

In Step III we picture the rate-limiting interconversion of the toxin-NAD-ribose-enzyme complex into a toxin-ADP-ribose-transferase II-nicotinamide complex. Steps IVa and b and V are the dissociation of the latter complex into the final products, toxin, ADP-ribose-transferase II, and nicotinamide.

The mode of inhibitory action proposed for adenine and NADH predicts that they should also inhibit the reverse reaction. NADH, in fact, does inhibit reversal. However, inhibition of reversal was not observed even by the highest concentration of adenine used (16 μM). The inability of adenine to inhibit the reverse reaction may be rationalized as follows. In the forward reaction, adenine (and NADH) competes with NAD for binding to toxin (Steps Ia, IIb, IIc) while, in the reverse reaction, it competes with ADP-ribose-transferase II (or ADP-ribose-transferase II-nicotinamide) for binding to toxin (Steps IVb, V). The inhibitory effectiveness of a given concentration of adenine is determined by the ratio of its association constant with that of the competing reaction. If ADP-ribose-transferase II (or ADP-ribose-transferase II-nicotinamide) binds to toxin with a higher association constant than does NAD then a given concentration of adenine will be less active in inhibiting the reverse reaction. The concentration of adenine needed to inhibit the reverse reaction may, in fact, exceed the solubility of adenine.

Our results show that GTP inhibits the reverse reaction. We cannot yet distinguish whether GTP acts in the reverse direction by inhibiting the binding of toxin to ADP-ribose-transferase II (Steps IVb and V) or by inhibiting the binding of nicotinamide to ADP-ribose-transferase II (Steps IVa and Vb). The latter possibility is favored since, in the forward direction, GTP inhibits competitively with respect to NAD. In either case, the model predicts that GTP can bind to ADP-ribose-transferase II. This is consistent with the report of Raeburn et al. (6) that prior treatment of partially purified transferase II with toxin and NAD does not reduce its ability to bind GTP.

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