Competitive Binding of Adenine and Nicotinamide-Adenine Dinucleotide to Diphtheria Toxin

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In a previous paper (Montanaro & Sperti, 1967) the binding of nicotinamide-adenine dinucleotides to diphtheria toxin was reported. The affinity of the toxin is higher for the reduced than for the oxidized form of the dinucleotides. Dissociation constants, calculated by means of fluorescence titrations at 0.01 *I*, pH 7.0 and 25°, were 7×10^{-7} M for NADH₂ and approx. 1×10^{-5} M for NAD.

A reversible binding of NAD to diphtheria toxin was also observed by Goor & Pappenheimer (1967). The dissociation constant of the toxin-NAD complex at 0.1 *I* and pH 7.5 was tentatively calculated to be 4×10^{-4} M by equilibrium dialysis.

In cell-free systems diphtheria toxin interferes with protein synthesis only in the presence of NAD (Collier & Pappenheimer, 1964), and the inhibition is due to inactivation of the enzyme transferase II involved in peptide bond-formation (Collier, 1967). It has been suggested that the actual active compound is a toxin-NAD complex (Montanaro & Sperti, 1967; Goor & Pappenheimer, 1967), which reacts with transferase II (Goor, Pappenheimer & Ames, 1967) to give an inactive product.

In the present communication evidence is presented that the adenine moiety of NAD is involved in the binding of the dinucleotide to diphtheria toxin.

Materials and methods. Purified diphtheria toxin containing 2.6 µg. of protein/Lf was obtained as previously described (Montanaro & Sperti, 1967). NAD, adenine and nicotinamide were products of Sigma Chemical Co. (St Louis, Mo., U.S.A.). [8-¹⁴C]Adenine (31.5 mc/m-mole) and [carbonyl-¹⁴C]nicotinamide (13.2 mc/m-mole) were products of The Radiochemical Centre, Amersham, Bucks., and were diluted with unlabelled carrier to a specific activity of 1 mc/m-mole.

Fluorimetric experiments were carried out in a C.G.A. model DC/3000 spectrofluorimeter (Dr A. Ciampolini, Florence, Italy) as described previously (Montanaro & Sperti, 1967). Sedimentation experiments were carried out at 4° in an MSE Superspeed 40 preparative centrifuge (8 × 10 ml. rotor). Samples (6 ml.) of 1 µM-toxin in 10 mM-tris-HCl buffer, pH 7.0, were centrifuged in the presence of various concentrations of [¹⁴C]adenine or [¹⁴C]nicotinamide (0.65–84 µM) at 105 000 g for 24 hr. By this time virtually all the toxin sedimented in the lower

layers. Free adenine or nicotinamide concentrations were calculated from the radioactivity of samples removed from the 0.5 ml. top layers and were corrected for the sedimentation of the radioactive compounds in control tubes centrifuged in the absence of toxin.

Equilibrium-dialysis experiments were performed in Plexiglas chambers separated by a cellophane disk cut from a Visking 20/32 tube (Visking Co., Chicago, Ill., U.S.A.). A 1 ml. sample of 10 µM-toxin solution in 10 mM-tris-HCl buffer, pH 7.0, was dialysed against 1 ml. of buffer. [¹⁴C]Adenine or [¹⁴C]nicotinamide was initially present at the same concentration (4–40 µM) in the two compartments. After continuous shaking for 24 hr. at 4°, radioactivity was measured on samples withdrawn from both compartments. Free adenine or nicotinamide concentration was calculated from the radioactivity of the compartment without toxin.

For radioactivity measurements 0.1 ml. of the aqueous samples were mixed in counting vials with 6 ml. of ethylene glycol monomethyl ether and 10 ml. of scintillation fluid [0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene], and were counted in a Nuclear-Chicago mark I scintillation counter (counting efficiency approx. 60%; background 22 counts/min.). The counts were corrected to 100% efficiency.

Results. (a) Fluorimetric experiments. When NAD (final concentration 24 µM) is added to 1 µM-toxin solution in 10 mM-tris-HCl buffer, pH 7.0, a 20% quenching of protein fluorescence (activation at 290 mµ, emission at 340 mµ) is observed (Montanaro & Sperti, 1967). The quenching caused by NAD was not affected by subsequent addition of nicotinamide, and stepwise addition of adenine to the toxin-NAD mixture caused an increase of protein fluorescence that approached the value for free toxin when the adenine concentration approximated 60 µM. Since in the absence of NAD adenine did not influence the fluorescence of toxin, the reversal of quenching observed on addition of adenine must result from a displacement of bound NAD due to a competitive binding of adenine at the same site on the toxin molecule (see Velick, 1958). From the fractional increment of protein fluorescence, the ratio between the dissociation constants of the two ligands could be calculated

Fig. 1. Binding of adenine to diphtheria toxin in the absence (●) and in the presence (■) of NAD. (a) Plot of $\bar{r}$ against $\bar{r}/c$ from sedimentation analysis of $1\mu\text{M}$ -toxin in the presence of increasing concentrations of adenine ($0.65\text{--}84\mu\text{M}$). The equation for the straight line, calculated by the least-squares method, was $\bar{r}=0.86-0.34\times 10^{-5}\bar{r}/c$. (b) Plot of $1/\bar{r}$ against $1/c$ from equilibrium dialysis of $10\mu\text{M}$ -toxin in the presence of increasing concentrations of adenine ($4\text{--}40\mu\text{M}$). In the absence of NAD the equation for the straight line was $1/\bar{r}=1.19+1.59\times 10^{-5}/c$; in the presence of $80\mu\text{M}$ -NAD the equation was $1/\bar{r}=1.16+4.86\times 10^{-5}/c$.

according to the procedure of Velick (1958). The dissociation constants for NAD and for adenine so calculated were of the same order (10^{-5}M).

(b) Sedimentation analysis. A direct binding of adenine to diphtheria toxin was confirmed by sedimentation analysis. Though the presence of diphtheria toxin did not affect the sedimentation of nicotinamide, the amount of adenine sedimented in the presence of toxin was considerably higher than the amount in control tubes containing adenine alone. Fig. 1(a) shows the results of a plot of $\bar{r}$ against $\bar{r}/c$ obtained from two adenine-binding experiments. According to the equation (Hayes & Velick, 1954):

$$\bar{r} = n - K_d\bar{r}/c$$

where $\bar{r}$ is the number of adenine molecules bound/molecule of protein and c is the molar concentration of free adenine, the intercept on the ordinate axis is the maximal number of protein-binding sites, and the negative slope defines the dissociation constant. The results were somewhat uncertain because of the scatter of the experimental points.

(c) Equilibrium dialysis. This method provided more consistent data for the calculation of the dissociation constant of the adenine-toxin complex. The competition of adenine and NAD for the same binding site on the protein molecule was also tested. Fig. 1(b) shows a plot of $1/\bar{r}$ against $1/c$ in the

absence and in the presence of a constant concentration of NAD. According to the equation:

$$1/\bar{r} = 1/n + K_d/n c$$

in the absence of NAD the intercept on the ordinate axis is the reciprocal of stoichiometry, and the intercept on the abscissa axis is $-1/K_d$. In the presence of a competitive ligand the plot has the same intercept on the ordinate axis, but the slope increases by a factor equal to $(1 + [I]/K_i)$, where $[I]$ is the concentration and K_i the dissociation constant of the competitive ligand (Klotz, Triwush & Walker, 1948). In the absence of NAD n was 0.84 and K_d was $1.34\times 10^{-5}\text{M}$. In the presence of NAD n was 0.86 and K_i , calculated from the ratio between the two slopes, was $3.89\times 10^{-5}\text{M}$. No appreciable binding of nicotinamide to toxin could be detected by equilibrium dialysis.

Comment. The results reported in the present paper indicate that both adenine and NAD interact with diphtheria toxin with similar equilibrium constants and that the same site on the protein molecule is involved in the binding of the two substances. It therefore appears likely that NAD reacts with diphtheria toxin with its adenine moiety.

Goor *et al.* (1967) postulate that in cell-free systems the toxin-NAD complex inhibits protein

synthesis by reacting with the enzyme involved in peptide bond-formation, transferase II, according to one of the two alternative reactions:

Nicotinamide added to the system prevents and even reverses the inhibition of protein synthesis, with the release of free active transferase II (Goor *et al.* 1967).

Since the adenine moiety of NAD appears involved in the binding of the dinucleotide to diphtheria toxin, the reactivation of transferase promoted by nicotinamide suggests a competition of nicotinamide and NAD for the same site on the transferase II molecule. NAD would thus be present in the toxin-transferase complex (reaction 1

proposed by Goor *et al.* 1967) bound to the toxin with its adenine and to the enzyme with its nicotinamide group, acting as a bridge between the two proteins.

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