Binding of Nicotinamide-Adenine Dinucleotides to Diphtheria Toxin

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1. Changes in protein fluorescence have been utilized in determining the stoichiometry and dissociation constants of the complexes of diphtheria toxin with NADH₂, NAD, NADPH₂ and NADP. 2. The binding stoichiometry is 2 moles of NADH₂ and 1 mole of NADPH₂/mole of diphtheria toxin. The binding sites for NADH₂ appear to be equivalent and independent. 3. The toxin shows a higher affinity for the reduced than for the oxidized forms of the nucleotides. 4. Dissociation constants at 0.01 *I*, pH 7 and 25° are 0.7×10^{-6} M for NADH₂ and 0.45×10^{-6} M for NADPH₂. Dissociation constants increase with increasing ionic strength, indicating that the binding is mainly electrostatic. 5. Bound NADH₂ and NADPH₂ may be activated to fluoresce by the transfer of energy from the excited aromatic amino acids of the toxin. Activation and emission spectra of bound and free nucleotides are compared. 6. Since NAD and NADH₂ are cofactors specifically required for the inhibition of protein synthesis by diphtheria toxin, the possible role of toxin-nucleotide complexes is discussed in this regard.

Diphtheria toxin inhibits protein synthesis by interfering with the transfer of amino acids from transfer RNA to the growing polypeptide chain (Collier & Pappenheimer, 1964). Since in cell-free amino acid-incorporating systems the inhibitory effect of the toxin occurs only in the presence of NAD or NADH₂, it has been suggested (Collier & Pappenheimer, 1964) that the toxin may activate a latent NAD-requiring enzyme that interferes with the transfer reaction; alternatively, diphtheria toxin, activated by NAD, may itself be an enzyme able to act on some essential component required for amino acid transfer.

In the present paper evidence of binding of NADH₂ to diphtheria toxin is presented. The stoichiometry of the complex, the dissociation constant and the spectral shifts of bound NADH₂ have been studied by fluorescence spectroscopy. Most of the experiments done with NADH₂ were also carried out with NADPH₂.

MATERIALS AND METHODS

Apparatus. A C.G.A. model DC/3000 spectrofluorimeter (Dr A. Ciampolini, Florence, Italy) was used. The instrument, equipped with an arrangement for a reference phototube to operate an automatic servo regulatory slit, produces a constant excitation energy. The excitation spectra are thus partially corrected. No correction was applied to emission spectra. The instrument was modified to contain a thermostatically controlled cuvette holder. The temperature inside the cuvette was checked by means of a small NTC (negative temperature coefficient) resistor

(Philips E205CE/P15K). The potential difference across the resistance thermometer was compared with that across a standard resistor by means of a Beckman model G potentiometer. Temperature was measured with an accuracy of 0.05°. Fluorescence intensity was read on the centesimal scale of the microammeter provided with the instrument. Emission was brought on scale by adjusting the gain of the photodetector. The gain was held constant whenever a set of curves had to be compared.

Chemicals. Diphtheria toxin, purified by the method of Pope & Stevens (1958), was obtained from the Istituto Sieroterapico e Vaccinogeno Toscano 'Sclavo' (Siena, Italy). The final product contained 2.6 µg. of protein/Lf, 1 Lf being that amount of toxin which gives the fastest flocculation with 1 standard unit of antitoxin in the Ramon test (see Wilson & Miles, 1964). NAD, NADP, NADH₂ and NADPH₂ were products of Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Fluorimetric titrations. The binding of nucleotides to diphtheria toxin was followed by measuring the decline in protein fluorescence at 340 mµ (activation at 290 mµ) on addition of the nucleotide. The quenching of protein fluorescence at 340 mµ is a reflection of the binding of the nucleotide by the protein and allows calculation both of stoichiometry and of dissociation constants (Vestling, 1962). Diphtheria toxin was dissolved (72 µg./ml.) in 10 mM-tris-HCl buffer, pH 7.0. NADH₂ and NADPH₂ in the same buffer were approx. 0.15 mM for the experiments at 0.01 *I*, and 0.30 mM at higher ionic strengths; NAD and NADP were 0.60 mM. Ionic strength was increased by addition of NaCl to the 10 mM-tris-HCl buffer. The exact concentration of the nucleotide solutions was determined from the extinction at 340 mµ (6.22×10^6 cm.² mole⁻¹) or at 260 mµ (18×10^6 cm.² mole⁻¹). Titrations were performed by adding a total of 0.3 ml. of nucleotide solution to 2.5 ml.

of toxin solution. The nucleotide was delivered in increments of 0.002–0.02 ml. from a Agla micrometer syringe (Burroughs Wellcome and Co., London, N.W.1). Samples were stirred after each nucleotide addition by means of a Teflon rod inserted through the top of the cell housing, and were shielded from incident light when readings were not being taken. All titration data were corrected for dilution.

In some instances complex-formation was followed by measuring nucleotide fluorescence at 480 m μ . Incident light was at 290 m μ and activation occurred by energy transfer from the protein (Velick, 1958).

RESULTS

Quenching of protein fluorescence: stoichiometry and dissociation constant. Fluorescence intensity was plotted against molar concentration of added nucleotide for each titration and the plot was corrected for beam attenuation as described by Velick, Parker & Eisen (1960). Typical titration curves are shown in Fig. 1. Controls in which diphtheria toxin was titrated with a dichromate solution having the same extinction at 290 m μ as the reduced nucleotide showed no fluorescence quenching, but only a constant slope due to beam attenuation.

The end points of the titration curves shown in Fig. 1 indicate a 1:2 stoichiometry for the diphtheria toxin–NADH₂ complex and a 1:1 stoichiometry for the diphtheria toxin–NADPH₂ complex. Stoichiometry was checked as described by Stockell (1959) by using equations derived by Klotz (1946) for the binding of ions to proteins. For n equivalent and independent binding sites a plot of d/p_1 as ordinate against $1/(p-p_1)$ as abscissa gives a straight line according to the equation:

$$\frac{d}{p_1} = \frac{K_d}{p-p_1} + n$$

where d is the total molar concentration of nucleotide, p is the total molar concentration of protein and p_1 is the molar concentration of bound protein. p_1 is obtained from fluorimetric data as described below. Plots according to Stockell (1959) are shown in Fig. 2. Slope and ordinate intercept were calculated by the method of least squares. The values of the ordinate intercept n (i.e. the number of independent protein binding sites) were 1.94 and

Fig. 1. Fluorescence titrations of diphtheria toxin with NADH₂ and NADPH₂. The toxin concentration was 72 μ g./ml. of 10 mM-tris-HCl buffer, pH 7.0. Assuming a molecular weight of 72 000 for diphtheria toxin (Relyveld & Ben Efraim, 1959), the end points of the titration curves indicate a 1:2 stoichiometry for the toxin–NADH₂ complex and a 1:1 stoichiometry for the toxin–NADPH₂ complex. The experimental curves (●) were corrected for beam attenuation (----). The maximal quenching (Q_{max}) by NADH₂ was 40.5% and by NADPH₂ 47.5%. From the corrected curves Q_i values were obtained and used for the calculation of the dissociation constant at each point. The average K_d was 0.672×10^{-6} M for NADH₂ and 0.411×10^{-6} M for NADPH₂. On the right is shown a control experiment (○) in which the nucleotide was replaced by a dichromate solution having the same extinction at 290 m μ . No quenching of fluorescence was observed, but only a constant slope due to beam attenuation.

Fig. 2. Plots of the data from the titrations of diphtheria toxin with NADH_2 (●) and with NADPH_2 (○) according to Stockell (1959). The least-squares slopes, i.e. K_d , were $0.744 \times 10^{-6} \text{ M}$ for NADH_2 and $0.450 \times 10^{-6} \text{ M}$ for NADPH_2 . The ordinate intercepts were 1.94 and 0.84, indicating that 1 mole of diphtheria toxin combines with 2 moles of NADH_2 and 1 mole of NADPH_2 .

0.84 for NADH_2 and NADPH_2 respectively. These values confirm the stoichiometry indicated by the end points of the titration curves. The slopes of the graphs shown in Fig. 2 give the values of the average intrinsic dissociation constants, K_d .

K_d can be independently calculated from the data at each point of the titration curves. The proportion of protein bound at each point is the ratio between the observed quenching at that point (Q_i) and the quenching of the protein saturated with nucleotide (Q_{max}) (Eisen & Siskind, 1964). The ratio Q_i/Q_{max} , multiplied by the total molar concentration of protein gives the concentration of bound protein (p_1). On the assumption of equivalent and independent binding sites, the intrinsic dissociation constant, K_d , for each point is calculated according to the relationship:

$$K_d = \frac{c(p-p_1)}{p_1}$$

where $p-p_1$ is the concentration of free protein and c is the concentration of free nucleotide computed on the basis of 2 protein binding sites for NADH_2 and 1 protein binding site for NADPH_2 .

Fig. 3. Heterogeneity plots. Slope and intercept were calculated by the method of least squares. The slopes yield a heterogeneity index of 0.943 for NADH_2 (●) and of 1.040 for NADPH_2 (○). The average association constant, K_0 , calculated from the intercept, was $1.502 \times 10^6 \text{ M}^{-1}$ for NADH_2 and $2.360 \times 10^6 \text{ M}^{-1}$ for NADPH_2 .

The presence of equivalent and independent binding sites on the protein molecule is indicated by the linearity of the plots in Fig. 2. Moreover, the value of the dissociation constant calculated at each point on the titration curve remained fairly constant throughout the curve; only in those regions where less than 10% or more than 90% of the protein had reacted did some variations occur. The variations were, however, at random, and apparently due to the fact that the error of the method is greatest in these regions of the curve. The data from these regions were not included in the calculation of the average equilibrium constant for a given titration. The dissociation constants obtained by adding and averaging the constants calculated at each point of the titration curves are reported in Table 1 and are in good agreement with the average constants calculated from the slopes of Fig. 2.

Association constant and heterogeneity index. The average intrinsic association constant, K_0 , i.e. the reciprocal of the average intrinsic dissociation constant, K_d , was calculated independently by using the equation:

$$\log \left(\frac{r}{n-r} \right) = a \log c + a \log K_0$$

Table 1. *Stoichiometry and equilibrium constants of NADH₂ and NADPH₂ complexes with diphtheria toxin at pH7 and 25°*

The results are expressed as means $\pm$ s.e.m. with the numbers of titrations given in parentheses. Average K_d and stoichiometry from Stockell plots are those of the experiments reported in Fig. 2.

	$10^6 \times$ average K_d from titration curves (M)	$10^6 \times$ average K_d from Stockwell plots (M)	Stoichiometry from titration curves	Stoichiometry from Stockell plots	$10^{-6} \times K_0$ from heterogeneity plots (M ⁻¹)	α (hetero- geneity index)
NADH ₂	0.725 ± 0.022 (9)	0.744	2.03 ± 0.025 (9)	1.94	1.519 ± 0.053 (7)	0.948 ± 0.054 (7)
NADPH ₂	0.452 ± 0.100 (14)	0.450	1.12 ± 0.033 (14)	0.84	2.080 ± 0.157 (7)	0.932 ± 0.041 (7)

Fig. 4. Temperature-dependence of the dissociation constants of the diphtheria toxin-NADH₂ complex (●) and of the diphtheria toxin-NADPH₂ complex (○). T is absolute temperature and $\ln K_d$ is the natural logarithm of the average dissociation constant.

derived from the generalized adsorption isotherm as defined by Sips (see Eisen & Siskind, 1964). In the above equation r is moles of nucleotide bound/mole of diphtheria toxin at free nucleotide concentration c , and n is the number of protein binding sites. Since the plot of $\log[r/(n-r)]$ against $\log c$ gives a straight line, K_0 can readily be calculated from the ordinate intercept. The slope α is the heterogeneity index, which describes the dispersion of association constants about the average value K_0 . When $\alpha=1$ all sites have the same association constant; decreasing values of α correspond to increasing heterogeneity with respect to association constants. Fig. 3 shows heterogeneity plots. Mean values of the heterogeneity index, calculated from several experiments, are shown in Table 1. The values, very close to 1, indicate a small distribution of association constants around the average constant. The reciprocals of the average association constants agree well with the dissociation constants calculated directly from the titration curves.

Thermodynamic parameters. The standard free energy (ΔG^0) of dissociation calculated from the average equilibrium constant is, at 25°, 8.36 kcal. mole⁻¹ for NADH₂ and 8.65 kcal. mole⁻¹ for NADPH₂. Fig. 4 shows Arrhenius-type plots of

the dissociation constants as a function of temperature. The absence of any appreciable slope from the curves indicates a small enthalpy contribution. ΔH^0 was calculated to be approx. 0.40 kcal. mole⁻¹ for NADH₂ and 1.70 kcal. mole⁻¹ for NADPH₂. According to these results, the entropy and not the enthalpy factor is dominant in the strong binding of NADH₂ and NADPH₂ to diphtheria toxin. The values of ΔS^0 at 25° are approx. -27 e.u. and -23 e.u. for the dissociation of NADH₂ and NADPH₂ respectively.

Effect of ionic strength. Titrations of diphtheria toxin with NADH₂ and NADPH₂ were carried out in the presence of increasing concentrations of sodium chloride in the 10mm-tris-hydrochloric acid buffer. The dissociation constants increased with increasing ionic strength. At 0.1M-sodium chloride the dissociation constant was approx. 2.4×10^{-6} M for NADH₂ and 2.6×10^{-6} M for NADPH₂. At 1.0M-sodium chloride the dissociation constants are so high that the binding of the two nucleotides has practically no effect on protein fluorescence.

Binding of NAD to diphtheria toxin. NAD, which has no absorption peak in the 340m μ region, can cause quenching of protein fluorescence when combined in a complex (Velick, 1958). A quenching of diphtheria toxin fluorescence by NAD was observed, but only at nucleotide concentrations much higher than those used in titrating the toxin with NADH₂. This indicates a much larger dissociation constant of the diphtheria toxin-NAD complex. Since titrations were carried out with nucleotide in large excess, and since the amount of NAD bound at any point was negligible, the concentration of free nucleotide could be assumed equal to that of added nucleotide. The dissociation constant was calculated by a double-reciprocal plot of $1/p_1$ versus $1/d$ as suggested by Velick (1958). The intercept on the abscissa axis, $-1/K_d$, was calculated by the method of least squares. K_d was 1×10^{-5} M in 10mm-tris-hydrochloric acid buffer, pH7.0. K_d for the binding of NADP, calculated by the same method, was 2×10^{-5} M.

Activation of nucleotide fluorescence by energy transfer. The addition of reduced nucleotide causes a considerable quenching of diphtheria toxin fluorescence activated at 290m μ and measured at

Fig. 5. Nucleotide fluorescence activated by energy transfer from the protein. NADH_2 (●) and NADPH_2 (○) fluorescence was activated at $290\text{m}\mu$ and read at $480\text{m}\mu$. Diphtheria toxin concentration was $1\mu\text{M}$. The solid lines are the theoretical curves for K_d (NADH_2) = $0.7 \times 10^{-6}\text{M}$ and for K_d (NADPH_2) = $0.45 \times 10^{-6}\text{M}$. The intercepts of the extrapolated initial and final slopes indicate that 1 mole of diphtheria toxin binds 2 moles of NADH_2 and 1 mole of NADPH_2 .

Fig. 6. Activation and emission spectra of free NADH_2 (----) and of NADH_2 bound to diphtheria toxin (—). Protein emission was subtracted. NADH_2 concentration was in both cases $1.8\mu\text{M}$ in 10mM -tris-HCl buffer, pH 7.0, at 25° . Diphtheria toxin concentration was $1.0\mu\text{M}$. Emission spectra were activated at $340\text{m}\mu$.

$340\text{m}\mu$. At the same time fluorescence appears at $480\text{m}\mu$, i.e. at the emission maximum of the reduced nucleotides. This energy transfer from the protein to the bound nucleotide is utilized in the

Fig. 7. Activation and emission spectra of free NADPH_2 (----) and of NADPH_2 bound to diphtheria toxin (—). Protein emission was subtracted. NADPH_2 concentration was in both cases $0.9\mu\text{M}$ in 10mM -tris-HCl buffer, pH 7.0, at 25° . Diphtheria toxin concentration was $1.0\mu\text{M}$. Emission spectra were activated at $340\text{m}\mu$.

titrations shown in Fig. 5, where activation is set at $290\text{m}\mu$ and nucleotide fluorescence is read at $480\text{m}\mu$. Stoichiometry and dissociation constants obtained from the curves are similar to those obtained after the quenching of protein fluorescence at $340\text{m}\mu$. Fig. 5 shows that the maximal fluorescence reached when the protein is saturated with nucleotide is much higher for the toxin- NADPH_2 complex than for the toxin- NADH_2 complex.

Activation and emission spectra of free and bound nucleotides. Figs. 6 and 7 illustrate the effect of complex-formation on the emission and activation spectra of NADH_2 and NADPH_2 . Fluorescence spectra were activated in the $340\text{m}\mu$ absorption band of reduced nucleotides. The binding to diphtheria toxin enhanced considerably the fluorescence of NADPH_2 and shifted its maximum from 475 to $450\text{m}\mu$. The maximum of NADH_2 was also shifted towards shorter wavelengths, whereas its fluorescence intensity was only slightly affected. Activation spectra were read at $480\text{m}\mu$. No appreciable shift of the $340\text{m}\mu$ excitation maximum was observed for the bound nucleotides. The maximum at $270\text{m}\mu$ of free nucleotides was instead shifted in both complexes approx. $10\text{m}\mu$ towards longer wavelengths, and was considerably enhanced, particularly with the toxin- NADPH_2 complex. This strong activation peak in the $280\text{m}\mu$ region probably arises by transfer of excitation energy

from the protein to the bound nucleotide (Velick, 1958). A correspondence is observed between the intensity of the 280m μ activation maximum of the two complexes and their fluorescence in the 480m μ region resulting from energy transfer.

DISCUSSION

The present studies clearly indicate that diphtheria toxin binds both NADH₂ and NADPH₂. The values of the dissociation constants are not dissimilar from those observed in the binding of NADH₂ and NADPH₂ to specific apodehydrogenases (Shifrin & Kaplan, 1960; Vestling, 1962). The changes of fluorescence spectra of NADH₂ and NADPH₂ in the presence of diphtheria toxin are also qualitatively similar to those observed in the presence of NAD-requiring apodehydrogenases. With these proteins the spectral changes observed are a shift of the maximal emission of NADH₂ towards shorter wavelengths when activated at 340m μ and an increased intensity of the fluorescence emission (Winer, Schwert & Millar, 1959). In the interaction of NADH₂ and NADPH₂ with diphtheria toxin both effects are present, but are most evident with NADPH₂. This suggests a more rigid orientation of bound NADPH₂ than of bound NADH₂, resulting in an increased probability for the bound nucleotide to lose activation energy by fluorescence rather than by collision (Shifrin & Kaplan, 1960). In the complexes of reduced nucleotides with diphtheria toxin a transfer of activation energy occurs from the aromatic amino acids of the protein to the bound nucleotides. This is indicated (see Velick, 1958) by the production of nucleotide fluorescence by excitation at 290m μ in the presence of diphtheria toxin and by the appearance in this region of a strong peak in the activation spectra of bound nucleotides. These effects are also more evident with the toxin-NADPH₂ complex.

Both complexes are very sensitive to ionic strength, indicating that the binding is mainly electrostatic in nature.

The association of NADH₂ and NADPH₂ with diphtheria toxin is accompanied by a large decrease in free energy. Since the enthalpy change is very small, the binding is associated with a large net increase of the entropy of the system. As suggested by Klotz & Urquhart (1949), the increase in entropy that accompanies the association of ions to proteins may be attributed to a decrease in hydration at the binding sites.

Collier & Pappenheimer (1964) have clearly shown that in cell-free systems diphtheria toxin inhibits protein synthesis only in the presence of a cofactor identified as NAD or NADH₂. NADP

added as a cofactor is only 1–2% as effective as NAD. To provide an explanation of the specific requirement for NAD, Collier & Pappenheimer (1964) postulated that the toxin either is a NAD-requiring enzyme or may activate some latent NAD-requiring enzyme that interferes with protein synthesis. The results reported in the present paper support the first hypothesis in that inhibition of protein synthesis may result from a binding of NAD to diphtheria toxin. It is, however, not necessary to conclude that the toxin-NAD complex acts as an enzyme. Since, according to our results, diphtheria toxin also binds NADP, the different effects of the two nucleotides in conferring sensitivity to toxin on cell-free protein-synthesizing systems may result from stoichiometric and configurational differences between the two complexes, differences that are clearly indicated by titration and spectral data. It is noteworthy that the lack of linearity between inhibition of protein synthesis and the amount of NAD added to a constant amount of diphtheria toxin (Collier & Pappenheimer, 1964) disappears if the results are interpreted on the basis that the actual active compound is a 2:1 toxin-NAD complex with an apparent dissociation constant of about 10⁻⁵M, i.e. not very dissimilar from that calculated from fluorimetric data.

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