# Histidine 21 Is at the NAD+ Binding Site of Diphtheria Toxin*

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Treatment of fragment A chain of diphtheria toxin (DT-A) with diethylpyrocarbonate modifies His-21, the single histidine residue present in the chain, without alteration of other residues. Parallel to histidine modification, NAD+ binding and the NAD-glycohydrolase and ADP-ribosyltransferase activities of DT-A are lost. Both NAD+ and adenosine are very effective in protecting DT-A from histidine modification and in preserving its biological properties, while adenine is ineffective. Reversal of histidine modification with hydroxylamine restores both NAD+ binding and enzymatic activities of the toxin.

The possible role of His-21 in the activity of diphtheria toxin is discussed in relation to the available three-dimensional structure of the related toxin produced by *Pseudomonas aeruginosa.*

Diphtheria toxin (DT),[1] a protein toxin secreted by pathogenic strains of *Corynebacterium diphtheriae*, is the agent responsible for most clinical symptoms of diphtheria (1). The active form of DT is composed of two polypeptide chains: A (21.164 kDa) and B (37.194 kDa) (2–4).

DT binds to the cell surface with the B chain and is internalized via receptor-mediated endocytosis. As the pH becomes acidic in the endosomal lumen the A chain of the toxin, in an as yet unknown manner, crosses the membrane and reaches the cytoplasm (5–8). Here it catalyzes the transfer of ADP-ribose from NAD+ to elongation factor 2 which becomes inactive with consequent block of cell protein synthesis (2).

The enzymatic activity of DT has been studied in detail (9–13). It has been shown that its A chain is an ADP-ribosyltransferase as well as an NAD+-glycohydrolase and that it binds NAD+ at a single site with a $K_D$ of ~8 $\mu$M. There is evidence that Lys-39, Gly-52, Gly-128, and Glu-148 are involved in the enzymatic function of DT-A (14–17). Sequence comparisons with the exotoxin A produced by *Pseudomonas aeruginosa* (ETA), another toxin that ADP-ribosylates elongation factor 2 (18–22), show that the segment Tyr-His-Gly-Thr is conserved. This segment lies in a cleft, present in the 3.0-Å map of the catalytic domain of ETA (21, 23), that is

thought to be involved in NAD+ binding (22, 23). His-21 of DT is present in the tetrapeptide segment and is the sole histidine residue present in the A chain of DT, while 15 such residues are present in its B chain (3, 4).

To test the possibility that His-21 of DT is involved in NAD+ binding, we have here used diethylpyrocarbonate (DEPC), a reagent that converts histidine to *N*-carbethoxyhistidine (24–26). Such treatment causes a specific modification of the single histidine of DT-A with a parallel loss of NAD+ binding and ADP-ribosyltransferase and NAD+-glycohydrolase activities.

## EXPERIMENTAL PROCEDURES

*Materials*—DT was obtained and purified as described by Rappuoli *et al.* (27). The toxin was nicked by trypsin treatment (12) and stored at −80 °C in 10 mM sodium phosphate, pH 7.4, at concentrations of 1–7 mg/ml. Elongation factor 2 was isolated from rat liver (28). Diethylpyrocarbonate was purchased from Aldrich (Italy); its titer was determined before use as described (26). L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, NAD+, adenine, adenosine, nicotinamide, fluorescamine, trinitrobenzenesulfonic acid, and iodoacetamide were from Sigma (Federal Republic of Germany). [adenine-2,8-³H]NAD+ (specific activity 33.4 Ci/mmol) was from Du Pont-New England Nuclear (F.R.G.). [carbonyl-¹⁴C]NAD+ (specific activity 44 mCi/mmol) was from Amersham (United Kingdom).

*Fragment A Isolation*—DT-A was obtained from nicked DT by incubation for 1 h at 37 °C in 50 mM Tris/Cl buffer, pH 7.0, containing 1 mM EDTA, 50 mM DTT, 8 M urea and separation on a Sephadex G-100 column eluted with 50 mM Tris/Cl, pH 8.2, 1 mM EDTA, 4 M urea, 2 mM DTT. Fractions containing DT-A were pooled and treated with 5 mM iodoacetamide for 15 min at 25 °C. The reaction was stopped with 30 mM β-mercaptoethanol, and the solution was dialyzed at 4 °C against 20 mM NaP$_i$, pH 7.4, 0.5 mM EDTA. Fragment A appeared as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis run as before (29). Its NAD+-glycohydrolase and ADP-ribosyltransferase activities were equal to that of an equivalent amount of DTT-treated DT.

*Modification of DT-A with DEPC*—The reaction was performed at a protein concentration of 0.2 mg/ml in 50 mM sodium phosphate, pH 7.8, at 25 °C by adding aliquots of a freshly prepared solution of DEPC in anhydrous ethanol; final concentration of ethanol never exceeded 2% (v/v). When required, the reaction was stopped by addition of imidazole, final concentration 5 mM. Histidine modification was reversed by incubation with 40 mM hydroxylamine-HCl, pH 7.8. In some experiments NAD+, adenosine, adenine, and nicotinamide were present at the concentrations reported in the figure legends. In this case histidine modification, NAD+ binding, and enzymatic activities were measured after removal of ligands by dialysis at 4 °C against 50 mM sodium phosphate, pH 7.0.

The time course of DT-A modification at histidyl and tyrosyl residues was followed by simultaneous recording of the differential absorbance increase at 243 nm and the differential absorbance decrease at 278 nm as described (25, 26). Modified residues were estimated with differential extinction coefficients of 3200 cm⁻¹ M⁻¹ at 243 nm for *N*-carbethoxyhistidine (24) and a differential extinction coefficient of −1310 cm⁻¹ M⁻¹ at 278 nm for *O*-carbethoxytyrosine (25) in a Perkin-Elmer Lambda 5 spectrophotometer.

*Assay of Free Amino Groups*—DT-A or DEPC-treated DT-A was modified with fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione) as described in Ref. 30. Briefly, to 10 $\mu$g of

[1] The abbreviations used are: DT, diphtheria toxin; DT-A, fragment A of diphtheria toxin; ETA, exotoxin A of *P. aeruginosa*; DEPC, diethylpyrocarbonate; fluorescamine, 4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione; DTT, dithiothreitol.

*NAD Binding Site of DT*

protein, dissolved in 1.25 ml of 50 mM sodium phosphate, 0.15 M NaCl, pH 7.5, 0.25 ml of 10 mM fluorescamine in acetone was added under stirring at room temperature. The extent of primary amino group modification was determined after 10 min at 475 nm (excitation wavelength of 390 nm) (31); five different samples were examined. Toxin amino groups were also assayed by treatment with trinitrobenzenesulfonate (32). To 30 μg of protein in 90 μl of 50 mM sodium phosphate, pH 7.8, 90 μl of 4% NaHCO₃, pH 8.5, and 90 μl of 0.1% trinitrobenzenesulfonate were added. The solution was kept in the dark for 2 h at 40 °C, and, after addition of 90 μl of 10% sodium dodecyl sulfate and 45 μl of 1 M HCl, its absorbance at 340 nm was measured and an extinction coefficient of $1.4 \times 10^4$ cm$^{-1}$ M$^{-1}$ was used to calculate the number of modified primary amino groups (32); four different samples were examined.

*NAD⁺ Binding*—The binding of NAD⁺ to control or DEPC-modified DT-A was measured both by tryptophan fluorescence quenching and flow dialysis according to Ref. 13. Briefly, to 1.5 ml of DT-A (2–4 μg/ml) in 50 mM NaP₁, pH 7.8, at 25 °C aliquots of NAD⁺ (2 μl) were added under stirring, and fluorescence was read at 330 nm in a Perkin-Elmer 650-40 fluorometer (excitation at 280 nm) and corrected for dilution and beam attenuation by titrating in parallel a solution of tryptophan. The relative fluorescence decrements, proportional to the binding of NAD⁺ to DT-A, were analyzed by Scatchard plots to obtain the maximal fluorescence decrement corresponding to maximal NAD⁺ binding. For flow dialysis measurements an apparatus composed of two cylindric chambers (10 mm diameter; upper chamber, 1 ml, lower chamber, 0.1 ml), separated by a dialysis membrane, was used. 200 μl of DT-A (0.95 mg/ml) in 50 mM NaP₁, pH 7.8, 10 μM [carbonyl-¹⁴C]NAD⁺ were placed in the upper chamber and the same buffer without NAD⁺ was passed through the lower chamber (flow rate, 1 ml/min), and 1-ml fractions were collected. 1-μl aliquots of NAD⁺ were then added to the upper chamber every six fractions. 0.7 ml of each fraction was mixed with 4 ml of Instagel (Packard) and counted with a Tri-Carb 300C liquid scintillation counter.

*NAD⁺-glycohydrolase Activity*—DT-A (0.1 mg/ml) or DT (0.3 mg/ml), previously activated by a 30-min treatment with 50 mM DTT, was incubated at 37 °C in 50 mM sodium phosphate, 6 μM [carbonyl-¹⁴C]NAD⁺, pH 7.8. At different times aliquots of 200 μl were removed and mixed with 50 μl of 5 M KCN, and the radioactive hydrolyzed nicotinamide was extracted with 2 ml of water-saturated ethyl acetate. 1.5 ml of the ethyl acetate phase was mixed with 10 ml of Instagel (Packard) and counted as above.

*ADP-ribosyltransferase Activity*—DT-A (0.1–0.5 μg/ml) or DTT-activated DT (0.3–1.5 μg/ml) was incubated at 25 °C with rat liver elongation factor 2 in 50 mM sodium phosphate, 6 μM NAD⁺, pH 7.8, containing 4.4 μCi/ml [adenine-2,8-³H]NAD⁺. At different times, 50-μl aliquots at the mixture were removed and mixed with 1 ml of 5% trichloroacetic acid. The trichloroacetic acid-precipitated material was filtered on a cellulose filter (0.45 μm pore diameter, Millipore) and, after extensive washing with 5% trichloroacetic acid, the filters were dried, incubated for 12 h in 10 ml of Instagel, and counted as above.

## RESULTS AND DISCUSSION

Sequence comparisons (16, 19–22) and an analysis of the available three-dimensional structure of the related ETA toxin (21, 23) suggested that His-21 of DT may play an important role in the function of DT. Of the 16 histidine residues present in DT, only 1, His-21, is present in the enzymatically active fragment A. Preliminary experiments showed that it is difficult to selectively modify His-21 with DEPC in the intact toxin. Hence, fragment A was purified and used in all subsequent DEPC modification experiments.

Fig. 1A shows the time dependence of the modification of DT-A with DEPC (80 μM), followed by the increase in absorbance at 243 nm, diagnostic of formation of N-carbethoxyhistidine. The rate of reaction declines rapidly due to hydrolysis of DEPC (24–26); an addition of fresh DEPC (80 μM) causes further modification, while further additions are without effect. From the absorbance spectra reported in Fig. 1B, it can be estimated that 0.95 histidine residues/A chain have reacted after 20 min. Fig. 1A shows the reversal of DEPC modification brought about by hydroxylamine (*dotted line*),
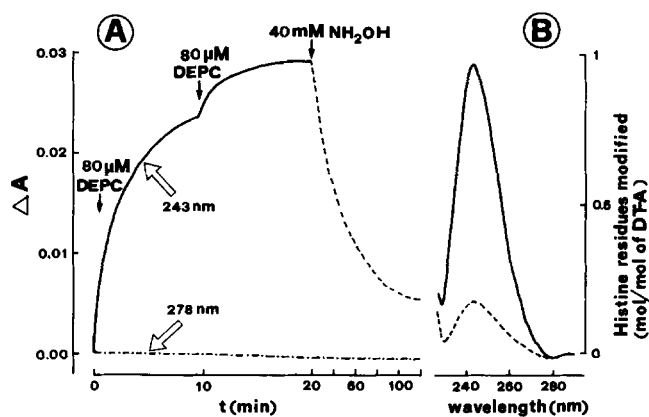


FIG. 1. **Time course of the modification of DT-A with DEPC.** *A*, absorbance changes at 243 and 278 nm of a solution of DT-A (0.2 mg/ml in 50 mM sodium phosphate, pH 7.8, at 25 °C) made 80 μM in DEPC at time 0. After 10 min an equivalent amount of DEPC was added; after 20 min hydroxylamine was added to a final concentration of 40 mM. Differential absorbances at 243 nm (—) and 278 nm (---) were measured with a double-wavelength program in a Perkin-Elmer Lambda 5 spectrophotometer. *B*, corresponding differential spectra of DT-A treated with DEPC for 20 min (—) and with hydroxylamine (---) as in *A*. A scale indicating the estimated number of modified histidine per DT-A molecule is shown on the *right side* of *B*.

which acts rapidly on N-carbethoxyhistidine but not on O-carbethoxytyrosine (25, 26). From Fig. 1A it also appears that no tyrosine residues are modified, under the conditions used here, because there is no change of absorbance at 278 nm indicative of formation of O-carbethoxytyrosine. In light of the recent demonstration that Lys-39 is at or near the NAD⁺ binding site of DT-A (16), it was important to determine if DEPC had modified lysine residues. Both fluorescamine and trinitrobenzenesulfonate assays of free amino groups of DT-A and DEPC-treated DT-A (0.9–0.96 histidines modified per A chain) showed the same amount of free amino groups. It could also be shown by fluorescence analysis that no tryptophan had reacted with DEPC (not shown).

Fig. 2 shows that both the NAD⁺ binding (assayed fluorimetrically) and the NAD⁺-glycohydrolase activity of DT-A are lost in parallel to DEPC treatment. It is noteworthy that the same result was obtained when NAD⁺ binding was assayed by flow dialysis (not shown). Together with the finding that the tryptophan fluorescence spectrum of DT-A does not change upon histidine modification, the result suggests that His-21 is not contiguous with the tryptophan residue quenched by NAD⁺.

The nearly complete restoration of NAD⁺ binding and enzymic activity of DT-A after hydroxylamine treatment is a further indication that a specific histidine modification is involved, because hydroxylamine rapidly cleaves N-carbethoxyhistidine, but slowly cleaves O-carbethoxytyrosine and does not react with DEPC-derivatized lysine (33).

The parallel effects of DEPC on histidine modification and on NAD⁺ binding could result either from His-21 being at or near the NAD⁺ binding site of DT-A or from a structural rearrangement of DT-A induced by DEPC modification. If the former possibility is correct NAD⁺ should be able to protect DT-A from modification with DEPC.

Fig. 3 shows the kinetics of DT-A reaction with DEPC as determined by its residual NAD⁺ binding, which is inversely correlated to the amount of modified histidine titrated spectrophotometrically after reaction with different concentrations of DEPC in the presence or in the absence of NAD⁺.

FIG. 2. **Loss of NAD⁺ binding and NAD⁺-glycohydrolase activity of DT-A treated with DEPC.** DT-A was treated with DEPC and hydroxylamine as described for Fig. 1*A*. At different times aliquots of the solution were removed, imidazole added to 5 mM and analyzed for NAD⁺ binding by fluorescence quenching and for NAD⁺-glycohydrolase activity. *Points* are the average of three different experiments run in duplicate, and *bars* represent ±S.D. Data are expressed as percent of the control value, taken as 100%.
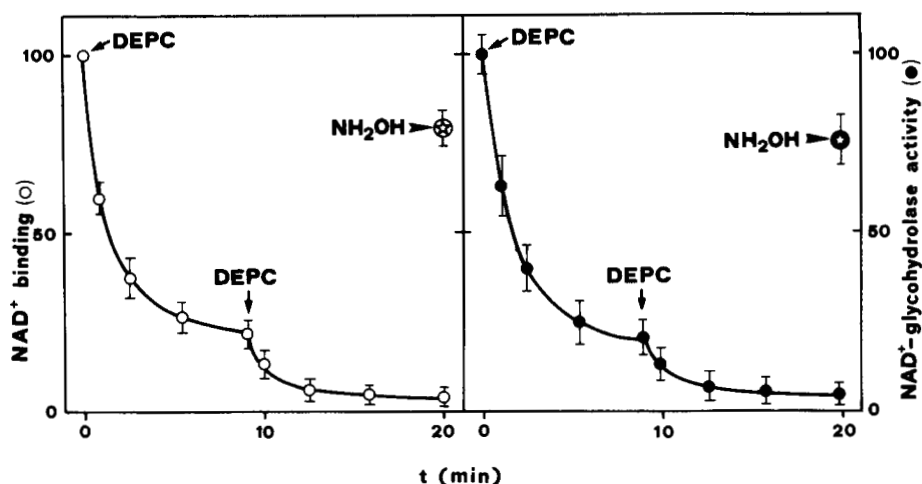
FIG. 3. **Protective effect of NAD⁺ from the DEPC modification of DT-A.** DT-A was modified with increasing concentrations of DEPC in the absence (*A*) or in the presence of 0.25 mM NAD⁺ (*B*). At different times aliquots of the mixture were removed, made 5 mM in imidazole diluted 100-fold in phosphate buffer, pH 7.8, and NAD⁺ binding was assayed fluorimetrically. Each point corresponds to the average of three independent sets of experiments run in duplicate. Standard deviation bars, here omitted for clarity, are in the range of those of Fig. 2. Data are referred to control binding values taken as 100%.
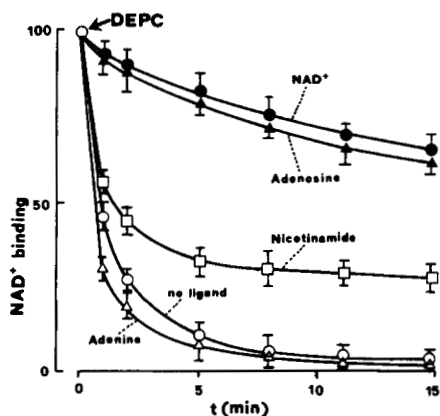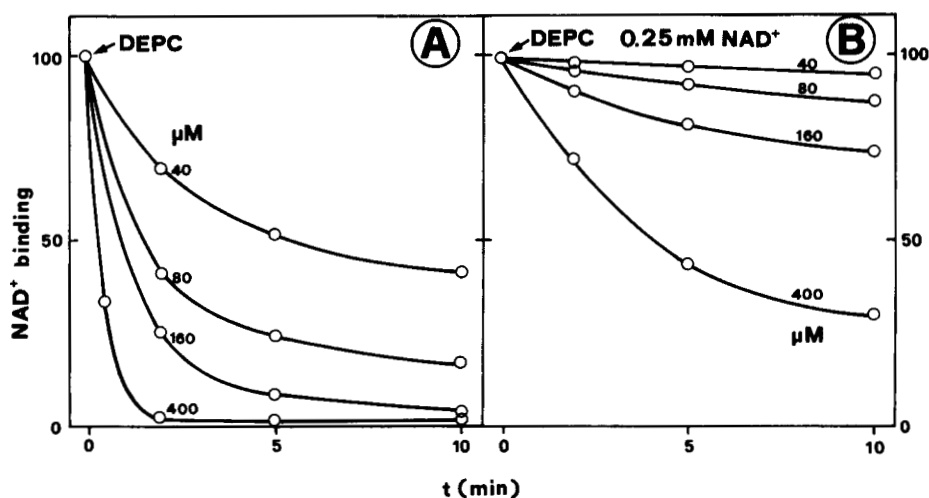
FIG. 4. **Protective effect on NAD⁺ binding of DT-A of various inhibitors with respect to DEPC modification.** The effect on DT-A NAD⁺ binding of treating DT-A with 160 $\mu$M DEPC in the presence of NAD⁺ (0.25 mM) of some inhibitors of NAD⁺ binding and activity of DT-A: adenine, 0.75 mM, adenosine 7 mM, and nicotinamide, 5.7 mM. These ligand concentrations give 96% saturation of the NAD⁺ binding site, according to their $K_D$ values (13). The reaction was performed as described for Fig. 1, and maximal NAD⁺ binding was measured at different times as described for Fig. 3. *Points* are the average of three separate experiments run as duplicates, and *bars* represent ±S.D.

Clearly, NAD⁺ is effective in protecting DT-A from DEPC modification.

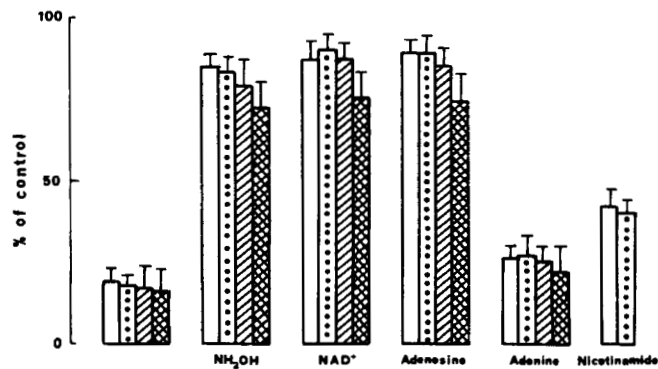Several moieties of the NAD⁺ molecule act as inhibitors of

FIG. 5. **Effect of DEPC treatment on different properties of DT-A in the presence of various agents.** DT-A or DT-A and ligands (concentrations as in Fig. 4) were incubated with 160 $\mu$M DEPC for 3 min, blocked with 5 mM imidazole or further incubated with hydroxylamine (40 mM for 1 h), dialyzed against NaP$_i$ (50 mM), pH 7.0, and assayed for histidine modification (spectrophotometrically), NAD⁺ binding (by fluorescence quenching), and NAD⁺-glycohydrolase and ADP-ribosyltransferase activities. Reported values are the average of three different experiments, run in duplicate, and are expressed as percentages of the control value. *Bars* represent ±S.D. □, residual His; ▣, NAD⁺ binding; ▨, NAD⁺ glycohydrolysis; ⊠, ADP-ribosyltransferase.

NAD⁺ binding to DT-A with different potencies: adenine ($K_D \sim 30\ \mu$M), nicotinamide ($K_D \sim 220\ \mu$M), and adenosine ($K_D \sim 270\ \mu$M) (13). It was therefore unexpected to find that the stronger ligand adenine is ineffective in protecting the NAD⁺

binding site of DT-A from DEPC modification, while the weaker ligand adenosine is nearly as effective as NAD⁺ (Fig. 4). The different protective effects of adenosine and adenine are not related to the different concentrations used to achieve 96% of saturation binding because adenine was ineffective even when present at the same concentration as adenosine.

Fig. 5 compares the effect of ligands on histidine modification, NAD⁺ binding, and enzymic activities. Any condition in which histidine is modified leads to a proportional loss of substrate binding with consequent loss of biological activity. Adenosine-protected DT-A is, within experimental error, as active as NAD⁺-protected or NH₂OH-treated DT-A both in NAD⁺-glycohydrolase and in ADP-ribosyltransferase activities.

Adenine and adenosine would be expected to influence DT-A structure in the same way. Hence, their different protective effect argues against the possibility that His-21 is distant from the NAD⁺ binding site and has its reactivity toward DEPC controlled by long range structural changes triggered by ligand binding.

Fig. 5 also shows that the loss of activity of DEPC-treated DT-A is not the result of a denaturation of the protein because both NAD⁺ binding and enzymatic activities are restored upon treatment with hydroxylamine.

Mutagenesis, proteolysis, and photoaffinity labeling experiments have provided evidence that Lys-39, Gly-52, Gly-128, and Glu-148 are involved in the catalytic function of DT (14–17, 34). Taken together, the results presented here provide strong evidence that His-21 is present at the NAD⁺ binding site of DT. Moreover, they suggest that His-21 may be very close to the sugar moiety of adenosine.

Very recently, Brandhuber *et al.* (21) have fitted adenosine, AMP, and ADP to the three-dimensional structure of ETA to map its NAD⁺ binding site. All residues proposed to be at the NAD⁺ binding site of DT on the basis of biochemical experiments (namely His-21, Lys-39, Gly-52 and Glu-148) are conserved in ETA and are at or near its NAD⁺ binding site as identified by x-ray diffraction methods (21). Moreover, the ETA map His-440, which corresponds to His-21 of DT, projects its imidazole ring near to the adenine-linked sugar ring, as suggested for DT by the present results.

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