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Tyrosine 65 Is Photolabeled by 8-Azidoadenine and 8-Azidoadenosine at the NAD Binding Site of Diphtheria Toxin*

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8-Azidoadenine and 8-azidoadenosine, two photoactivatable derivatives of adenine and adenosine, are competitive inhibitors of diphtheria toxin of similar potency with respect to their parent compounds. On irradiation, the two tritium-labeled photoactivatable azidoadenines bind covalently and specifically to an enzymic fragment of diphtheria toxin that is known to bind to NAD. This photolabeling is protected by the enzyme substrate NAD. The radiolabeled protein was fragmented, and the radioactive fragments were sequenced. Tyr-65 is labeled specifically by both photoreagents, and its labeling was reduced strongly when NAD was present during irradiation. Labeling is also reduced strongly by adenine, adenosine, and nicotinamide. These results suggest that Tyr-65 is at the NAD binding site of diphtheria toxin and that the competitive inhibitors adenine, adenosine, and nicotinamide bind to the same portion of the catalytic center of the toxin.

Diphtheria toxin belongs to a family of bacterial protein toxins that catalyze the transfer of ADP-ribose from NAD to an intracellular target (Pappenheimer, 1977; Collier, 1982; Rappuoli and Pizza, 1990). They have the common structural architecture of being formed by two parts, A and B, joined by a single interchain disulfide bridge. Protomer B is responsible for cell binding and also for the translocation of protomer A across the membrane in the case of diphtheria toxin (Montecucco *et al.*, 1990). Chain A of these toxins is endowed with both ADP-ribosyltransferase and NAD-glycohydrolase activities (Collier, 1982).

Protomer A of diphtheria toxin may be isolated after trypsin and dithiothreitol treatment of the toxin, and it is composed of 193 residues (Ratti *et al.*, 1983; Greenfield *et al.*, 1983). It binds NAD at a single site with high affinity ($K_d \sim 8$ μM), whereas portions of the NAD molecule act as competitive inhibitors: adenine ($K_d \sim 30$ μM), nicotinamide ($K_d \sim 220$ μM), and adenosine ($K_d \sim 270$ μM) (Kandel *et al.*, 1974).

Chain A of diphtheria toxin recently has been crystallized in different crystal forms (Kantardjieff *et al.*, 1989), but its structure is not yet available. There is evidence that Glu-148, Lys-39, and His-21 are present at the NAD binding site of diphtheria toxin (Carroll and Collier, 1984; Tweten *et al.*, 1985; Zhao and London, 1988; Papini *et al.*, 1989, 1990). The determination of the residues involved in NAD binding and in enzymic function is important, not only for an understanding of the mechanism of catalysis, but also for the design of mutant forms of diphtheria toxin to be evaluated as potential candidates for new vaccines against diphtheria.

To identify other residues forming the NAD site of diphtheria toxin, we have used [2-^{3}H]8-azidoadenine and [2-^{3}H]8-azidoadenosine, two photoactivatable analogues of the competitive inhibitors of diphtheria toxin adenine and adenosine. These compounds are stable in the dark, but irradiation with ultraviolet light converts them into the corresponding nitrene derivatives. These are highly reactive intermediates that are able to form covalent bonds with neighboring molecules, thus labeling radioactively amino acid residues present at their binding site (Bayley, 1983; Montecucco, 1988).

EXPERIMENTAL PROCEDURES

Materials—Diphtheria toxin was purified from culture filtrates of *Corynebacterium diphtheriae* according to Rappuoli *et al.* (1983); nicked by trypsin treatment (Drazin *et al.*, 1971); and, after freezing in liquid nitrogen, stored at −80 °C in 10 mM K-Hepes,[1] 0.2 mM EDTA, pH 7.2. Exotoxin A from *Pseudomonas aeruginosa* was obtained by the Swiss Vaccine Institute (Berne, Switzerland). L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, NAD, adenine, adenosine, 8-azidoadenosine, nicotinamide, iodoacetamide, and *N*-chlorosuccinimide were from Sigma (Germany). 2-(2-Nitrophenylsulfenyl)-3-bromo-3-methylindolenine and *o*-iodosobenzoic acid were from Pierce (Holland). *p*-Cresol was from Fluka (Switzerland). *Staphylococcus aureus* V8 protease was a product of Boehringer Mannheim. [*carbonyl*-^{14}C]NAD$^+$ (specific activity, 50 mCi/mmol) was from Amersham Corp. [2-^{3}H]8-Azidoadenosine (specific activity, 20 Ci/mmol) was from Moravek Biochemicals (Brea, CA). 8-Azidoadenine and [2-^{3}H]8-azidoadenine were prepared by acid hydrolysis of the corresponding adenosine derivatives as described by Maliarik and Goldstein (1988).

Isolation of Chain A of Diphtheria Toxin—Nicked diphtheria toxin (30 mg/ml) was incubated for 1 h at 37 °C in 50 mM Tris/Cl buffer, pH 7.0, containing 1 mM EDTA, 50 mM dithiothreitol, 8 M urea, and gel filtered at 4 °C on a Sephacryl 200 column in 50 mM Tris/Cl, 1 mM EDTA, 4 M urea, 2 mM dithiothreitol, pH 8.2. Fractions containing protomer A of diphtheria toxin were pooled and treated with 5 mM iodoacetamide for 15 min at 25 °C in the dark. The reaction was terminated with 30 mM β-mercaptoethanol, and the solution was dialyzed at 4 °C against 10 mM K-Hepes, 0.2 mM EDTA, pH 7.4. Fragment A of the toxin, a single band on SDS-PAGE, was frozen as aliquots in liquid nitrogen and stored at −80 °C at a protein concentration of 1–2 mg/ml.

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[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-bromo-3-methylindolenine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

NAD-glycohydrolase Activity—Chain A of diphtheria toxin (100 μg/ml) was incubated in the dark at 25 °C in 50 mM sodium phosphate, pH 7.4, with 6 μM [*carbonyl*-^{14}C]NAD$^+$, in the absence or in the presence of various concentrations of adenine, 8-azidoadenine, adenosine, and 8-azidoadenosine. At different times, 200-μl aliquots were removed, mixed with 50 μl of 1 M sodium borate, pH 9.0, to stop the reaction (Carroll and Collier, 1988a). The radioactive nicotinamide produced by hydrolysis was extracted with 1 ml of water-saturated ethyl acetate and counted with a Tri-Carb 300C liquid scintillation counter. The concentration of the indicated competitors that gave 50% inhibition of the NAD-glycohydrolase activity (IC_{50}) was then calculated (Rankin *et al.*, 1989). Under these conditions the reaction was linear for 60 min and led to the consumption of less then 10% of the initial NAD.

Affinity Photolabeling—Diphtheria toxin or its protomer A (1.5 mg/ml) were incubated in the dark for 10 min at 25 °C in K-Hepes, 0.1 mM EDTA, pH 7.4, or in 50 mM Tris-Cl, 0.1 mM EDTA, pH 7.4, containing 6 μCi/ml of [2-^{3}H]8-azidoadenosine or [2-^{3}H]8-azidoadenine, and irradiated with an UV lamp (UVSL-58 Mineralight, Ultraviolet Products, San Gabriel, CA) as described previously (Montecucco, 1988). In most experiments an irradiation time of 15 min was used. Some samples also contained different concentrations of NAD, adenine, adenosine, or nicotinamide. The radioactivity covalently bound to the protein was measured after SDS-PAGE (Papini *et al.*, 1987), visualization of the protein bands by precipitation of protein-bound SDS with K^+ ions, and slicing. The radioactivity associated with the slices was determined by liquid scintillation after a 12-h treatment at room temperature with Soluene tissue solubilizer (Packard) and further incubation for 12 h with Instagel (Packard).

Protein Cleavage—Chain A of diphtheria toxin or photoaffinity-labeled chain A (1.5 mg/ml) in 10 mM K-Hepes, 0.1 mM EDTA, pH 7.4, was treated with *S. aureus* V8 protease (1 ng/μg diphtheria toxinA) at 37 °C for 30 min. The reaction was terminated by addition of 71 μg/ml phenylmethylsulfonyl fluoride, and the protein was recovered by precipitation with trichloroacetic acid (final concentration, 6%). Only one fragment stable to proteolysis was obtained that began with residue 16 and ended, on the basis of its migration in SDS-PAGE in the presence of urea (Kadenbach *et al.*, 1983), with Glu-100 or Glu-105. A peptide beginning with residue 106, as identified by protein sequencing, was formed transiently and degraded further very rapidly.

The reaction with *o*-iodosobenzoic acid was performed according to Fontana *et al.* (1981) with minor modifications. 300 μl of protomer A of diphtheria toxin or photoaffinity-labeled fragment A (1.5 mg/ml) in K-Hepes, 0.1 mM EDTA, pH 7.4, was mixed with 1.2 ml of a solution of glacial acetic acid containing 16 mg/ml *o*-iodosobenzoic acid, 2.5 M guanidine HCl, 25 μl/ml *p*-cresol, and incubated for 24 h in the dark at 25 °C. The reaction was terminated by the addition of β-mercaptoethanol, and the final concentration was 180 mM.

Cleavage with *N*-chlorosuccinimide was performed according to Lischwe and Sung (1977). 300 μl of chain A of diphtheria toxin (1.5 mg/ml) in K-Hepes, 0.1 mM EDTA, pH 7.4, was mixed with 750 μl of a mixture containing 1 ml of glacial acetic acid, 1 ml of distilled water, 1 g of urea, 0.5 mg/ml *N*-chlorosuccinimide, and incubated for 30 min at 37 °C.

The cleavage with BNPS-skatole was carried out following the method of Martenson *et al.* (1977). 300 μl of BNPS-skatole (6.2 mg/ml in glacial acetic acid) were added to 100 μl of diphtheria toxin A chain (1.5 mg/ml) in 10 mM K-Hepes, 0.1 mM EDTA, pH 7.4. After 24 h in the dark at 37 °C, the reaction mixture was diluted with 2.4 ml of distilled water, and the excess reagent and its by-products were removed by two extractions with 7 volumes of ethyl acetate.

The protein fragments obtained with these procedures were diluted 7-fold with 6% trichloroacetic acid, incubated at room temperature for 1 h, centrifuged for 20 min at 16,000 rpm, and subjected to SDS-PAGE as discussed. After staining with Coomassie Blue, the yield of cleavage was estimated by comparing the area of the corresponding bands obtained by densitometric scanning with those of standards. The sizes of the different fragments were estimated by their electrophoretic migrations with the following proteins as standards: diphtheria toxin protomer A (21,154 Da), cytochrome *c* (11,700 Da), pertussis toxin S-5 (11,000 Da), cytochrome *c* CNBr-fragments (7,300 and 2,700 Da).

Protein Sequences—Protein fragments were separated by SDS-PAGE and blotted on polyvinylidene difluoride membranes (Immobilon, Millipore) according to the method of Matsudaira (1987). The bands of interest were cut and submitted to automatic Edman degradation with a gas-phase sequenator model 470A connected on-line with a phenylthiohydantoin-amino acid analyzer model 120, equipped with a Control data module model 900A (Applied Biosystems, Foster City, CA). Radioactivity released during each Edman degradation cycle was measured by liquid scintillation as above.

Computer Molecular Modeling—Model building was performed with three different programs: Mogli (Evans and Sutherland), Sybyl (Tripos Assoc.) and Quanta (Polygen), using their subroutines for the determination of distance geometry, the evaluation of the hydrogen-bonding, and the minimization calculations.

TABLE I

Comparison of the inhibition of the NAD-glycohydrolase activity of diphtheria toxin-A by different moieties of NAD and its azido derivatives at 25 °C

Values are means ± S.D.

Compound	IC_{50}	Relative IC_{50}
	mM	
Adenine	0.045 ± 0.005	1.0
8-Azidoadenine	0.050 ± 0.006	1.1
Adenosine	0.75 ± 0.02	16.7
8-Azidoadenosine	4.0 ± 0.1	88.9

RESULTS AND DISCUSSION

Adenine and adenosine are competitive inhibitors of the catalytic activities of diphtheria toxin (Kandel *et al.*, 1974). In the first set of experiments, we compared their inhibitor potency with those of their photoactivatable analogues 8-azidoadenine and 8-azidoadenosine by competing varying concentrations of each inhibitor against a fixed amount of NAD as described by Rankin *et al.* (1989). Table I compares the different inhibitors in terms of their IC_{50}, defined as the concentration that inhibits NAD-glycohydrolase activity of chain A of diphtheria toxin by 50% under the present conditions, and in terms of relative IC_{50}, obtained by dividing each IC_{50} by that of adenine. 8-Azidoadenine and 8-azidoadenosine are only slightly less potent inhibitors than their parent compounds as it is frequently found with photoreactive derivatives of purines (Bayley and Knowles, 1977; Bayley, 1983).

When diphtheria toxin was incubated in the presence of the two tritiated azidopurine derivatives, chain A was photolabeled specifically (not shown). In all subsequent experiments only chain A, the enzymic portion of diphtheria toxin, was used to obtain simpler protein fragmentation patterns (see below). Protomer A of diphtheria toxin incorporated radioactivity only when exposed to light, thus showing that there was no chemical labeling due to dark reactions. In preliminary experiments it was found that the amount of radioactivity associated with chain A under the present conditions reached a plateau with 10 min of ultraviolet irradiation. Thus, in all subsequent experiments an irradiation time of 15 min was used. Under these conditions there was no loss of NAD-glycohydrolase activity.

The amount of radioactivity incorporated in protomer A in the presence of 2 mM NAD was lowered in different experiments to an average 9% of the control without NAD, indicating that labeling occurred predominantly at the NAD binding site of the toxin. Closely similar results were obtained with the related exotoxin A from *P. aeruginosa*, which is photolabeled by [2-^{3}H]8-azidoadenine in an adenine-protected reaction.[2]

The average percentage of radioactivity covalently bound to chain A of diphtheria toxin was 28% with [2-^{3}H]8-azidoadenine and 0.8% with [2-^{3}H]8-azidoadenosine. The yield of photoinduced cross-linking can be defined as the fraction of reagent covalently bound after irradiation with respect to the

[2] G. Schiavo, E. Papini, and C. Montecucco, unpublished observations.

amount present at the NAD binding site of chain A. Taking into account the 9% unspecific labeling and the actual occupancy of the binding site at the photoreagent concentration of 0.3 μM, used in the labeling experiments, the yields of photolabeling were 44% for [2-^{3}H]8-azidoadenine and 42% for [2-^{3}H]8-azidoadenosine.

To locate the site(s) of photolabeling on the primary structure of chain A of diphtheria toxin we assayed different proteolytic methods and analyzed the results by SDS-PAGE in the presence of urea (Kadenbach *et al.*, 1983). This procedure allowed separation of the protein fragments on the basis of their molecular weights and provided a very efficient removal of residual noncovalently bound reagent. Chain A of diphtheria toxin was cleaved with *S. aureus* V8 protease which yielded only one stable fragment beginning with Asn-16 and ending, on the basis of the apparent molecular weight, with Glu-100 or Glu-105, which contained all the radioactivity incorporated by chain A. This fragment was sequenced up to residue 52 without release of any specifically radiolabeled amino acid derivative. This result indicates that the site of photolabeling was between residue 52 and residues 100 or 105.

To generate a fragment with a NH_2 terminus closer to the site of cross-linking, chain A of diphtheria toxin was cleaved at tryptophan residues, a procedure that resulted in the fragments outlined in Fig. 1. Of the three methods tested here, the best result was obtained with iodosobenzoic acid using the procedure of Fontana *et al.* (1981). Fig. 2*A* shows the densitometric profile of chain A after irradiation in the presence of [2-^{3}H]8-azidoadenine and cleavage with iodosobenzoic acid, and Fig. 2*B* shows the associated radioactivity. Fig. 2*B* also reports the pattern of labeling of a sample illuminated in the presence of 2 mM NAD. The yield of fragmentation at Trp-50 in different experiments was in the 40–45% range. Lower yields were obtained with BNPS-skatole (12–15%), whereas higher yields were found with *N*-chlorosuccinimide (54–57%). However, with this latter method a major part of the peptides had blocked NH_2 termini.

Fragments were identified by their apparent molecular weights in SDS-PAGE and by their sequence obtained after blotting onto Immobilon membranes. A very small amount of radioactivity was associated with fragments 1–50 and 154–193, and this amount was unaffected by the presence of NAD during irradiation. This result indicated that nonspecific labeling was very low and, together with the above described cleavage at carboxylic residues, that the site of photolabeling occurred within fragments 51–100(105).

Fragments 51–153 and 51–193 labeled with [2-^{3}H]8-azidoadenine or with [2-^{3}H]8-azidoadenosine, blotted onto Immobilon membranes, were sequenced in a gas-phase sequenator. The radioactivity released at each cycle was determined, and the result of a typical experiment with a [2-^{3}H]8-azidoadenosine-labeled fragment 51–193 is reported in Fig. 3. There

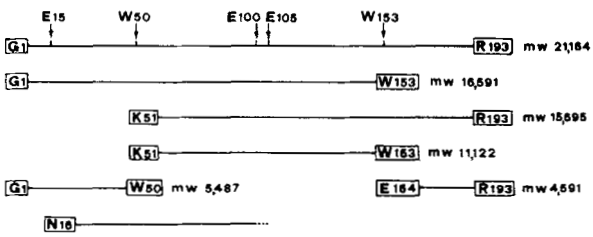

FIG. 1. **Scheme with molecular weights of protomer A of diphtheria toxin and of its fragments obtained by cleavage at tryptophans and carboxylic residues.** The COOH-terminal residues of the stable fragment generated by *S. aureus* V8 protease is estimated to be either at Glu-100 or at Glu-105 on the basis of migration in SDS-PAGE and inspection of the sequence.

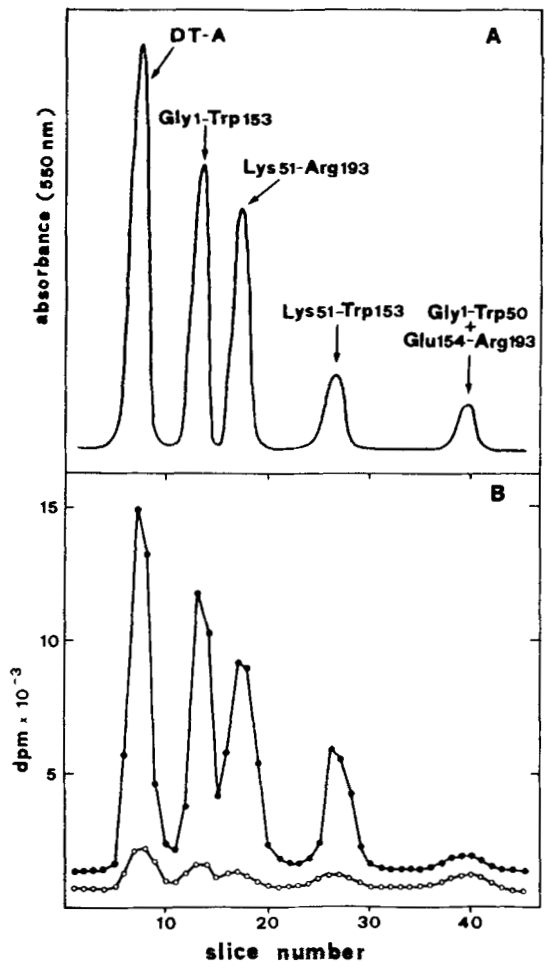

FIG. 2. **Pattern of labeling with [2-^{3}H]8-azidoadenine of the fragments originated by cleavage of chain A of diphtheria toxin with iodosobenzoic acid.** *A*, densitometric profile of a gel of chain A of diphtheria toxin (*DT-A*) irradiated in the presence of [2-^{3}H]8-azidoadenine, subjected to SDS-PAGE in the presence of urea, and stained with Coomassie Blue. *B*, distribution of the radioactivity associated to the same gel, determined after gel slicing and counting; the *open symbols* refer to a sample of chain A irradiated in the presence of 2 mM NAD.

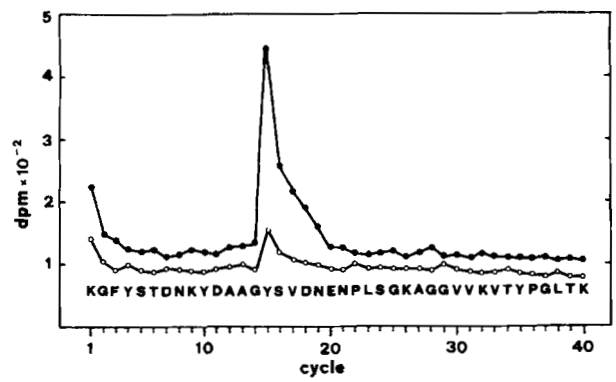

FIG. 3. **Determination of the site of photolabeling of [2-^{3}H] 8-azidoadenosine by protein sequencing.** The radioactivity released at each cycle of Edman degradation of fragment 51–193 (obtained as in Fig. 2), blotted onto Immobilon filters and placed in a gas-phase sequenator, is reported together with the identified amino acid residue. *Filled* and *open points* refer to samples irradiated in the absence and the presence of 2 mM NAD, respectively.

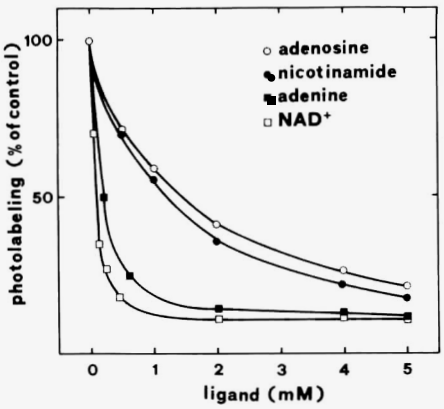

FIG. 4. **Inhibition of photolabeling of [2-^{3}H]8-azidoadenine to chain A of diphtheria toxin in the presence of varying concentrations of some competitive inhibitors of diphtheria toxin.** Chain A of diphtheria toxin was irradiated, as described under "Experimental Procedures," in the presence of [2-^{3}H]8-azidoadenine and of the reported concentrations of NAD, nicotinamide, adenine, and adenosine. The amount of radioactivity bound to the protein is reported as the percentage of that bound to chain A after irradiation in the absence of inhibitors. Data are the average of three or more determinations; S.D. bars are omitted for clarity and never exceeded 8%.

was a substantial increase of the background of radioactivity with respect to a nonirradiated sample from the first step, most likely due to acid hydrolysis of the photolabeled product, during the treatment with heptafluorobutyric acid. However, in all experiments performed either with the adenine or with the adenosine derivatives, the peak of radioactivity coincided with Tyr-65. After this release, radioactivity returned progressively to basal level within three to four cycles. Such a finding is observed frequently in sequencing photolabeled peptides (Ross *et al.*, 1982; Hoppe *et al.*, 1983; Giraudat *et al.*, 1986) and is due to an incomplete removal of the the labeled residue in a single cycle and to incomplete washing. No radioactivity was detected upon further sequencing up to residue 88. In different sets of experiments, it was estimated that the amount of radioactivity recovered before residue 68 was always higher than 85% of the starting amount.

As shown in Fig. 3, the peptide derived from chain A of diphtheria toxin photolabeled in the presence of NAD released a much lower amount of radioactivity with Tyr-65, as expected if NAD occupied the site of binding of the photoactive azidoadenosine. Closely similar results were obtained with 8-azidoadenine (not shown).

All together these data indicate that there is a unique site of specific photolabeling with [2-^{3}H]8-azidoadenine and [2-^{3}H]8-azidoadenosine at the level of Tyr-65 and that Tyr-65 is present at the NAD binding site of the A chain of diphtheria toxin. This result is in agreement with the finding of Brandhuber *et al.* (1988) that adenosine lies in the three-dimensional map of exotoxin A of *P. aeruginosa* very close to Tyr-481, which by sequence comparison (Brandhuber *et al.*, 1988; Carroll and Collier, 1988b; Zhao and London, 1988) is homologous to Tyr-65 of diphtheria toxin.

On the basis of this finding Brandhuber *et al.* (1988) proposed an arrangement of the NAD molecule with its adenosine moiety in the same position as that of free adenosine and the nicotinamide part of NAD lying over the indole ring of Trp-466 (Trp-50 of diphtheria toxin). This arrangement, however, does not account for the photoinduced cross-linking of the nicotinamide of NAD to Glu-148 of diphtheria toxin and to Glu-553 of exotoxin A (Carroll and Collier, 1984; Carroll and Collier, 1987), because the glutamic acid residue is too distant from nicotinamide.

To obtain more information on this point we determined the effect of different doses of various NAD site ligands on the amount of photoaffinity labeling of chain A with [2-^{3}H] 8-azidoadenine. Fig. 4 shows that not only NAD, adenine, and adenosine are potent competitors, but that nicotinamide also efficiently protects chain A of diphtheria toxin from photolabeling. The order of potency of the various ligands follows

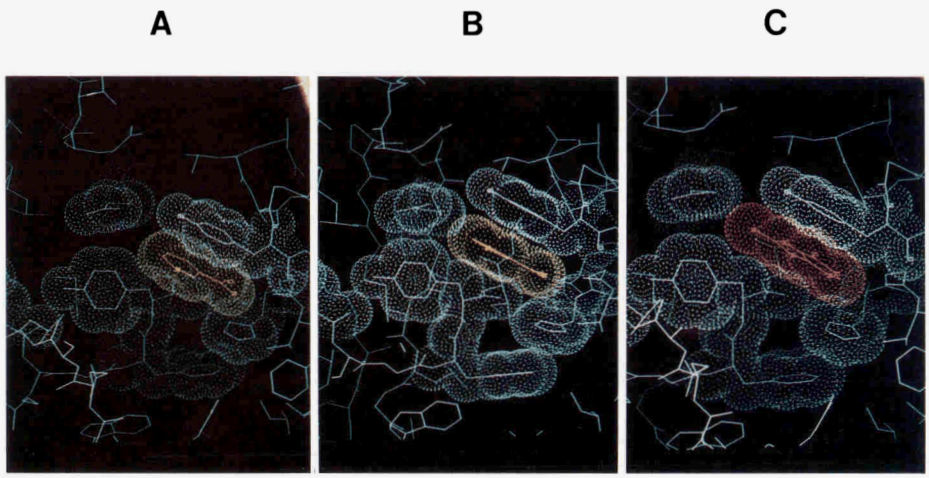

FIG. 5. **Molecular modeling of the NAD binding site of diphtheria toxin and molecular fitting of nicotinamide (*A*), adenine (*B*), and 8-nitrenoadenine (*C*).** The structure of the NAD binding site of diphtheria toxin was determined by computer modeling the primary structure of chain A of diphtheria toxin on the three-dimensional structure of the related exotoxin A from *P. aeruginosa* as described elsewhere.[3] Only the van der Waals radii of the atoms of residues forming the binding site are depicted. They are: *upper right*, Tyr-65; *right side*, His-21; *bottom*, Phe-53; *middle left*, Tyr-54; and *upper left*, Glu-148, Trp-50, also essential for NAD binding, can be identified easily by its indole ring at the *bottom*. By distance geometry the best fit for all three competitive inhibitors of diphtheria toxin was obtained when they lie with their aromatic cloud next to that of Tyr-65 with the aromatic rings exactly coplanar (see *panel A*). Additional hydrogen bonds may be formed, but localization of these aromatic molecules appears to be governed in each case by the aromatic stacking. Such an arrangement suggests that, on photolysis, a N–O bond may form between the nitrogen atom of the nitrene intermediate and the oxygen atom of the phenol group of Tyr-65.

the order of their binding affinities. This result suggests that nicotinamide and adenine share the same or closely spaced binding sites.

Fig. 5 shows pictures of the binding of nicotinamide, adenine, and 8-nitrenoadenine to chain A of diphtheria toxin obtained by a best fitting of their molecules in a molecular model of diphtheria toxin protomer A, obtained by replacing one by one the amino acid residues of exotoxin A of *P. aeruginosa* with the corresponding ones of chain A of diphtheria toxin on the atomic coordinates of the three-dimensional structure of exotoxin A (Allured *et al.*, 1986).[3] The best fit of all the three inhibitors was always obtained when their aromatic orbitals were stacked with those of the phenolic ring of Tyr-65. Localization of these aromatic molecules appears to be governed by this hydrophobic aromatic interaction and binding may be stabilized by additional hydrogen bonds. The orientation of the adenine molecule is nearly identical to that determined by x-ray diffraction methods for the exotoxin A of *P. aeruginosa* (Brandhuber *et al.*, 1988). In this way the nitrene atom in position 8 of the purine ring, formed by photoactivation of the corresponding azide, is very close to the OH of Tyr-65 in a optimal position to form a N–O covalent bond. The formation of such a derivative would explain the acid lability mentioned above.

The stacking of nicotinamide over Tyr-65 accounts for its inhibition of 8-azidoadenine photolabeling, reported in Fig. 4. Moreover it suggests the possibility that NAD binds to the catalytic center of diphtheria toxin and of exotoxin A in the opposite position with respect to the suggestion of Brandhuber *et al.* (1988), namely with its nicotinamide ring at the level of Tyr-65 of diphtheria toxin (Tyr-481 of exotoxin A) and the purine part over Trp-50 (Trp-466 of exotoxin A). Such a mode of NAD binding is discussed in detail elsewhere[3] and accounts well for the photolabeling of the nicotinamide moiety of NAD to Glu-148 of diphtheria toxin, because its carboxylate group lies next to position 2 of the nicotinamide ring of NAD. Moreover it is consistent with the yet unexplained finding that adenosine, which comprises a larger portion of NAD, is a weaker ligand than adenine.

REFERENCES

Allured, V. S., Collier, R. J., Carroll, S. F. & McKay, D. B. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1320–1324

Bayley, H. (1983) *Photogenerated Reagents in Biochemistry and Molecular Biology,* pp. 1–187, Elsevier Science Publishers, Amsterdam

Bayley, H. & Knowles, J. R. (1977) *Methods Enzymol.* **46,** 69–114

Brandhuber, B. J., Allured, V. S., Falbel, T. G. & McKay, D. B. (1988) *Proteins* **3,** 146–154

Carroll, S. F. & Collier, R. J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 3307–3311

Carroll, S. F. & Collier, R. J. (1987) *J. Biol. Chem.* **262,** 8707–8711

Carroll, S. F. & Collier, R. J. (1988a) *Methods Enzymol.* **165,** 218–225

Carroll, S. F. & Collier, R. J. (1988b) *Mol. Microbiol.* **2,** 293–296

Collier, R. J. (1982) in *ADP-Ribosylation Reactions* (Hayaishi, O., and Ueda, K., eds) pp. 575–592, Academic Press, New York

Drazin, R., Kandel, J. & Collier, R. J. (1971) *J. Biol. Chem.* **246,** 1504–1510

Fontana, A., Dalzoppo, D., Grandi, C. & Zambonin, M. (1981) *Biochemistry* **20,** 6997–7004

Giraudat, J., Dennis, M., Heidmann, T., Chang, J. Y. & Changeux, J. P. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2719–2723

Greenfield, L., Bjorn, M. J., Horn, D., Fong, D., Buck, G. A., Collier, R. J., and Kaplan, D. A. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 6853–6857

Hoppe, J., Montecucco, C. & Friedl, P. (1983) *J. Biol. Chem.* **258,** 2882–2885

Kadenbach, B., Jarausch, J., Hartman, R. & Merle, P. (1983) *Anal. Biochem.* **129,** 517–521

Kandel, J., Collier, R. J. & Chung, D. W. (1974) *J. Biol. Chem.* **249,** 2088–2097

Kantardjieff, K., Collier, R. J. & Eisenberg, D. (1989) *J. Biol. Chem.* **264,** 10402–10404

Lischwe, M. A. & Sung, M. T. (1977) *J. Biol. Chem.* **252,** 4976–4980

Maliarik, M. J. & Goldstein, I. J. (1988) *J. Biol. Chem.* **263,** 11274–11279

Martenson, R. E., Deibler, G. E. & Kramer, A. J. (1977) *Biochemistry* **16,** 216–221

Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038

Montecucco, C. (1988) *Methods Enzymol.* **165,** 347–357

Montecucco, C., Papini, E. & Schiavo, G. (1990) in *Structure, Regulation and Activity of Bacterial Toxins* (Alouf, J. E., and Freer, J. H., eds) Academic Press, London, in press

Papini, E., Schiavo, G., Tomasi, M., Colombatti, M., Rappuoli, R. & Montecucco, C. (1987) *Eur. J. Biochem.* **169,** 637–644

Papini, E., Schiavo, G., Sandoná, D., Rappuoli, R. & Montecucco, C. (1989) *J. Biol. Chem.*, **264,** 12385–12388

Papini, E., Schiavo, G., Rappuoli, R. & Montecucco, C. (1990) *Toxicon* **28,** 631–635

Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem.* **46,** 69–94

Rankin, P. W., Jacobson, E. L., Benjamin, R. C., Moss, J. & Jacobson, M. K. (1989) *J. Biol. Chem.* **264,** 4312–4317

Rappuoli, R., Perugini, M., Marsili, I. & Fabbiani, S. (1983) *J. Chromatogr.* **268,** 543–548

Rappuoli, R. & Pizza, M. (1990) in *Structure, Regulation and Activity of Bacterial Toxins* (Alouf, J. E. and Freer, J. H., eds) Academic Press, London, in press

Ratti, G., Rappuoli, R. & Giannini, G. (1983) *Nucleic Acids Res.* **11,** 6589–6595

Ross, A. H., Radhakrishnan, R., Robson, R. J. & Khorana, H. G. (1982) *J. Biol. Chem.* **257,** 4152–4161

Tweten, R. K., Barbieri, J. T. & Collier, R. J. (1985) *J. Biol. Chem.* **260,** 10392–10394

Zhao, J. M. & London, E. (1988) *Biochemistry* **27,** 3398–3403

[3] Domenighini, M., Montecucco, C., Ripka, W. C., and Rappuoli, R. (1991) *Mol. Microbiol.*, in press.