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Biochemistry

NAD binding site of diphtheria toxin: Identification of a residue within the nicotinamide subsite by photochemical modification with NAD

(photoaffinity labeling/ADP-ribosylation/exotoxin A/NAD-linked dehydrogenases)

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ABSTRACT **We showed earlier that exposing mixtures of NAD and diphtheria toxin fragment A to ultraviolet radiation (253.7 nm) induced the formation of covalently linked protein-ligand photoproducts. Here we report that when [*carbonyl*-^{14}C]NAD was employed in such procedures, the efficiency of labeling of the protein approached 1 mol/mol, and at least 94% of the incorporated label was associated with a single residue, glutamic acid at position 148. Fragment A photolabeled in this manner was enzymically inactive. The efficiency of photolabeling was much lower (<0.2 mol/mol) when NAD radiolabeled in either the adenine moiety or the adenylate phosphate was used, and the label was attached to different site(s) within fragment A. Efficient photochemical transfer of label from [*carbonyl*-^{14}C]NAD occurred under identical conditions with the nucleotide-free form of whole diphtheria toxin, CRM-45, or activated exotoxin A from *Pseudomonas aeruginosa*, but not with nucleotide-bound diphtheria toxin, CRM-197, native exotoxin A, or any of several NAD-linked dehydrogenases. On the basis of these and other results we suggest that part or all of the nicotinamide moiety of NAD is efficiently transferred to glutamate-148 of fragment A under the influence of ultraviolet irradiation and that this residue is located within the nicotinamide subsite. This location implies that glutamate-148 is at or near the catalytic center of the toxin. Our data provide direct evidence for the location of the NAD site in an ADP-ribosylating toxin and demonstrate highly efficient and specific photolabeling by [*carbonyl*-^{14}C]NAD.**

Several bacterial exotoxins act by catalyzing transfer of the ADP-ribose moiety of NAD into covalent linkage with specific target proteins of mammalian cells. For both diphtheria toxin (DT) and exotoxin A from *Pseudomonas aeruginosa* the target protein is elongation factor 2 (EF-2) (1). ADP-ribosylation inactivates the factor, thereby inhibiting protein synthesis and ultimately causing cell death. For cholera toxin and related enterotoxins the target is a subunit of the adenylate cyclase system (2). ADP-ribosylation enhances the activity of the cyclase, and the resulting increase in cAMP concentrations produces any of a variety of physiological changes, depending upon the type of cell affected. Recently, pertussis toxin has also been shown to ADP-ribosylate a component of adenylate cyclase (3).

DT (M_r, 58,342) is released from *Corynebacterium diphtheriae* as a single, 535-residue polypeptide of known primary structure (4, 5). The intact toxin is inactive in catalyzing the ADP-ribosylation of EF-2 and must be subjected to mild proteolysis and reduction for this activity to be expressed. Such treatment cleaves the toxin into an enzymically active A fragment (M_r, 21,167), and a B fragment (M_r, 37,195) involved in receptor recognition and membrane translocation.

In earlier studies we characterized the enzymic and ligand-binding properties of fragment A (6). The fragment was found to have a single NAD-binding site (K_d, 8 μM), which catalyzed not only the ADP-ribosylation of EF-2 but also the slow hydrolysis of NAD to ADP-ribose, nicotinamide, and H^+ (NAD glycohydrolase reaction; EC 3.2.2.5). Here we report results of photoaffinity labeling experiments designed to locate the catalytic center within the A fragment. Our data show that ultraviolet irradiation of mixtures of fragment A and NAD results in transfer of the nicotinamide moiety, or an altered form or portion thereof, to Glu-148. Both the efficiency of labeling (approaching 1 mol/mol) and the specificity of attachment are significantly greater than commonly observed with underivatized ligands (7–9) and suggest a mechanism possibly unique to the ADP-ribosylating toxins. The results presented support the notion that Glu-148 is at or near the catalytic center.

METHODS

Materials. Purification of monomeric DT (Connaught Laboratories, Toronto, ON, Canada) and fractionation into nucleotide-free and nucleotide-bound forms will be described elsewhere. Fragment A, derived from DT by mild proteolysis and reduction, was isolated by size-exclusion chromatography (6). The immunologically crossreactive proteins CRM-45 and CRM-197 were purified as described (10). *P. aeruginosa* exotoxin A was purified from culture fluids of strain PA-103 (11). Five times crystallized ovalbumin, lactate dehydrogenase (Calbiochem), α-chymotrypsin, carboxypeptidase A (Worthington), malate dehydrogenase (Miles), trimethylamine (Aldrich), and organic solvents (Burdick and Jackson, Muskegon, MI) were obtained from the sources indicated. Thermolysin, alcohol dehydrogenase, NAD, NAD-agarose, aminophenyl boronic acid-agarose, ADP-ribose, and cyanogen bromide were from Sigma. Trifluoroacetic acid, dansyl (Dns) chloride, Dns derivatives of amino acids, phenyl isothiocyanate, and phenylthiohydantoin derivatives of amino acids came from Pierce. All other reagents were of analytical grade.

The following isotopes were purchased from Amersham and repurified as necessary by chromatography on Dowex AG1-X2 (Bio-Rad) (12); [*carbonyl*-^{14}C]NAD (53 mCi/mmol), [*adenylate*-^{32}P]NAD (1000 Ci/mmol), and [*adenine*-^{14}C]NAD (501 mCi/mmol) (1 Ci = 37 GBq). Radioactivity was quantified in a Beckman LS-100 liquid scintillation counter with ACS counting fluor (Amersham) for ^{14}C-labeled isotopes or by measuring Cerenkov radiation for ^{32}P-labeled material.

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Abbreviations: DT, diphtheria toxin; EF-2, elongation factor 2; Dns, dansyl.

Photolysis. Ultraviolet-induced linking of native ligands to proteins was performed as follows (13): Samples in 50 mM Tris·HCl, pH 7.2, were applied as 100- to 150-μl droplets onto an inverted microtiter plate lid on ice and irradiated at a distance of 5.5 cm with a 15-W germicidal lamp (G15-T8; General Electric, Schenectady, NY). The irradiation was performed at low temperature to minimize NAD-glycohydrolase activity. At the times indicated, 20-μl aliquots were removed and added to 40 μl of ice-cold 3 M guanidine hydrochloride, and trichloroacetic acid-precipitable radioactive material was determined. For spectral analyses or enzymatic assays, photolyzed protein was separated from unreacted ligands and reaction by-products by chromatography on a 0.7 × 20 cm column of Sephadex G-50, equilibrated in 50 mM Tris·HCl, pH 7.2, at 4°C. Preparative samples were desalted on an identical column equilibrated in 5.7% acetic acid and lyophilized.

Assays. ADP-ribosyltransferase and NAD-glycohydrolase activities of DT and exotoxin A were analyzed by standard procedures (6). Absorption and difference spectra were determined in a Beckman model 25 spectrophotometer. The concentrations of DT ($A_1^{1\%}{}_{cm}$ = 13.4), fragment A ($A_1^{1\%}{}_{cm}$ = 15), and exotoxin A ($A_1^{1\%}{}_{cm}$ = 12) were determined spectrophotometrically at 280 nm. Other proteins and peptides were quantified fluorimetrically with *o*-phthalaldehyde (14).

Chemical and Enzymic Cleavage. Radiolabeled DT fragment A in 70% (wt/vol) formic acid was incubated for 24 hr at 25°C with a 100-fold molar excess of cyanogen bromide over methionine residues (15). Fifteen volumes of water was then added and the sample was lyophilized; the addition of water and subsequent lyophilization were twice repeated. The dried sample was redissolved in a minimal amount of 98% formic acid, diluted with 30% (vol/vol) acetic acid, and chromatographed on a 0.4 × 110 cm column of Sephadex G-75 fine in 30% acetic acid. The radiolabeled fractions were pooled, lyophilized, and digested with 2% thermolysin or chymotrypsin in 0.2 M *N*-ethylmorpholine acetate, pH 8.2. After 4–6 hr at 45°C or 37°C, respectively, digestion was terminated by the addition of glacial acetic acid.

Chromatographic Methods. For high-performance liquid chromatography (HPLC), we used a Waters system (16) equipped with a Bio-Rad 1305 variable ultraviolet absorbance detector. Enzymic digests were fractionated on an Alltech C_{18} reverse-phase column, or by chromatography on a 0.3 × 34 cm column of Dowex AG50W-X2 (Bio-Rad) in pyridine/acetate (17).

Amino Acid Analysis and Sequencing. NH_2-terminal analyses and compositional studies were performed using dansyl chloride, with chromatography of the dansyl derivatives on polyamide thin layers (18). Manual dansyl/Edman degradations followed the method of Allen (19). Manual Edman degradations were performed essentially as described by Tarr (20): Peptides were coupled with 10% phenyl isothiocyanate in 20 μl of trimethylamine/pyridine (2:1, vol/vol), washed with benzene, cleaved with trifluoroacetic acid, and extracted with benzene/ethyl acetate (2:1, vol/vol). Extracts were converted to phenylthiohydantoin derivatives in 1 M methanolic HCl and analyzed on high-performance thin-layer silica plates (21). Repetitive yields were generally greater than 97%.

RESULTS

Photoinduced Linking of NAD to Fragment A. We showed earlier that ultraviolet irradiation of fragment A in the presence of NAD induced the formation of photoproducts containing at least part of the dinucleotide covalently linked to the protein (13). Fig. 1 shows the results of an experiment designed to identify those portions of NAD that became attached to fragment A. Solutions of fragment A containing

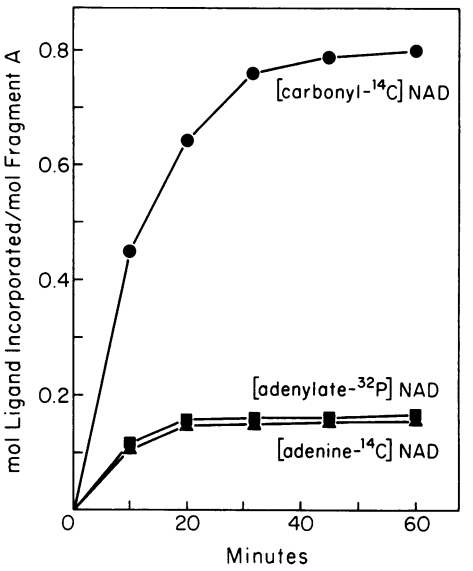

FIG. 1. Ultraviolet-induced labeling of fragment A in the presence of radiolabeled NAD. Reaction mixtures containing 50 mM Tris·HCl at pH 7.2, fragment A (20 μM), and NAD (40 μM) radiolabeled in the nicotinamide (●), the adenylate phosphate (■), or the adenine (▲) moiety were prepared at 4°C and irradiated with ultraviolet light (predominantly 253.7 nm). At intervals up to 60 min, aliquots were removed and denatured in guanidine hydrochloride, and the protein was precipitated by application to filter paper impregnated with 10% trichloroacetic acid. Acid-precipitable radioactivity was then determined after several washes in 5% trichloroacetic acid to remove unreacted ligand.

NAD radiolabeled in the adenine moiety, the adenylate phosphate, or the nicotinamide moiety, were irradiated under a low-pressure mercury lamp for up to 60 min, and trichloroacetic acid-precipitable radioactivity was determined. With nicotinamide-labeled NAD (carbonyl-^{14}C), both the rate and the extent of incorporation (0.67–0.93 mol/mol) were markedly higher than with either the adenine- or the phosphate-labeled dinucleotide (0.13–0.18 mol/mol).

When the nicked, nucleotide-free form of intact DT was substituted for fragment A, the photoinduced linking of nicotinamide-labeled NAD was again much greater than that observed with the dinucleotide labeled in other positions. The incorporated label migrated only with the A fragment during electrophoresis under reducing conditions in sodium dodecyl sulfate/polyacrylamide gels (data not shown). The efficiency of linking was less than 0.1 mol/mol when NAD was replaced with radiolabeled nicotinamide, either alone or in combination with unlabeled ADP-ribose.

The photoinduced incorporation of label from [carbonyl-^{14}C]NAD into other proteins under conditions effective for fragment A (20 μM protein, 40 μM NAD, 30 min) is shown in Table 1. Fragment A, the nucleotide-free form of DT, CRM-45, and activated *Pseudomonas* exotoxin A exhibited levels of incorporation much higher than those observed with other proteins. Moreover, the amounts of label incorporated into intact DT and activated exotoxin A were essentially identical, suggesting similarities in the nicotinamide subsites of these two exotoxins, the mechanism of labeling, or both. Increasing the concentrations of protein and NAD severalfold generally decreased the initial rate, but not the final extent, of linking, probably due to optical shielding caused by the higher concentrations of ultraviolet-absorbing reactants.

Enzymic Activity of Photolabeled Fragment A. After ultraviolet irradiation and removal of unreacted [*carbonyl*-^{14}C]NAD, radiolabeled fragment A was separated from the unlabeled fragment A by chromatography on a column of NAD-agarose (data not shown). Labeled material was unre-

Table 1. Ultraviolet-induced incorporation of label from [*carbonyl*-^{14}C]NAD into various proteins

Sample	mol % labeling*
DT fragment A	74
DT, nucleotide-free	39
DT, nucleotide-bound	12
CRM-45	36
CRM-197	5
Exotoxin A	5
Activated exotoxin A†	33
Alcohol dehydrogenase	6
Lactate dehydrogenase	5
Malate dehydrogenase	6
Ovalbumin	3

Each protein (20 μM) was irradiated at 0°C in 50 mM Tris·HCl, pH 7.2, containing 40 μM [*carbonyl*-^{14}C]NAD. After 30 min, acid-precipitable radioactivity was determined.
*(mol label incorporated/mol protein) × 100.
†Exotoxin A was activated by incubation with 4 M urea/15 mM dithiothreitol for 15 min at 25°C and then desalted by chromatography on a column of Sephadex G-50 equilibrated in 50 mM Tris·HCl, pH 7.2.

tarded, whereas unlabeled material bound and was subsequently recovered by elution with 10 mM NAD. Photolabeled fragment A exhibited negligible levels of NAD-glycohydrolase or ADP-ribosyltransferase activity compared with unlabeled material from the same photolysis mixture (Table 2). Consistent with earlier findings (13), the enzymic activities of the unlabeled material were lower than those of nonirradiated fragment A.

Isolation of Radiolabeled Peptides from Fragment A. Cleavage of photolabeled fragment A with cyanogen bromide and fractionation of the products on Sephadex G-75 yielded four peptide peaks (Fig. 2), similar to those described by Drazin *et al.* (15). Most of the label (73%) migrated with the third peak, which, on the basis of NH_2-terminal sequence analysis, corresponded to residues 116–178 of the primary structure (CNBr-3). The remainder was associated with higher molecular weight material migrating with uncleaved or partially cleaved A fragment. Reduction and carboxymethylation of photolabeled fragment A prior to cleavage with cyanogen bromide gave higher yields of labeled CNBr-3 (86%), whereas repeated treatment with cyanogen bromide had little effect.

After digestion of the radiolabeled CNBr-3 fraction with thermolysin, the peptide products were analyzed by reverse-phase HPLC at pH 2.0 (Fig. 3*a*). Sixty-seven percent of the label was associated with a single peak (Fig. 3*b*) eluting at 45 min. Rechromatography of the isolated peak at pH 4.5 yielded a single radiolabeled peptide. Thin-layer chromatography of this material after acid hydrolysis and reaction with dansyl chloride resolved three equimolar derivatives; Dns-Val,

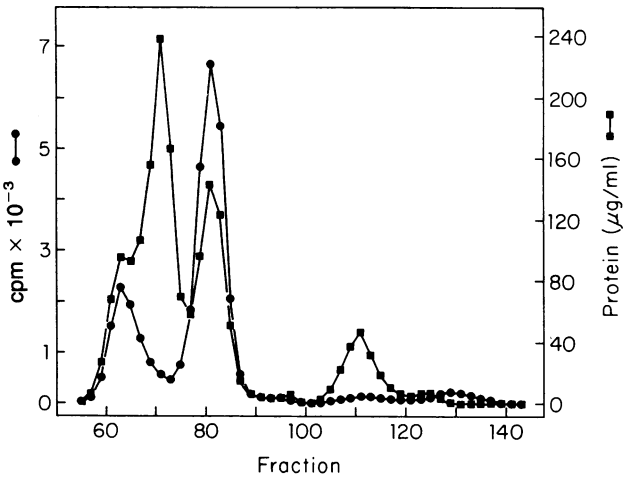

FIG. 2. Column chromatography of cyanogen bromide peptides from fragment A irradiated in the presence of [*carbonyl*-^{14}C]NAD. Mixtures of fragment A (80 μM) and nicotinamide-labeled NAD (160 μM) were irradiated at 0°C for 30 min, desalted on a column of Sephadex G-50 equilibrated in 5.7% acetic acid, and lyophilized. After resuspension in 70% formic acid, a 100-fold molar excess of cyanogen bromide over methionine residues was added, and the sample was incubated in the dark for 24 hr at 25°C. The reaction was terminated by dilution with 15 vol of water and lyophilization. Peptides were dissolved in 50 μl of 98% formic acid, diluted to 200 μl with 30% acetic acid, and chromatographed on a 0.5 × 110 cm column of Sephadex G-75 equilibrated with 30% acetic acid. Fractions (200 μl) were collected; 5-μl aliquots were removed for determination of radioactivity (●) and protein (■).

Dns-Tyr, and an unidentified residue migrating between Dns-Thr and Dns-Ser on polyamide. Valine was identified as the NH_2 terminus, while treatment with carboxypeptidase A liberated only tyrosine. By comparison with the known primary structure of fragment A (4, 5, 22), the radiolabeled material eluting at 45 min was tentatively identified as the unique tripeptide Val-Glu-Tyr (residues 147–149).

Two other thermolytic peptides containing most of the remaining radioactivity were resolved by reverse-phase HPLC at pH 2.0 (elution times 47 and 61 min; see Fig. 3*b*). These peptides were identified as partial digestion products (residues 147–156 and 140–149), both of which contained the Val-Glu-Tyr tripeptide.

Treatment of radiolabeled CNBr-3 with chymotrypsin followed by chromatography of the products on Dowex AG50W-X2, reverse-phase HPLC, or both gave the nonamer Ala-Glu-Gly-Ser-Ser-Ser-Val-Glu-Tyr (residues 141–149) as the only labeled component. Hydrolysates of this peptide contained 1 mol of Glx per mol instead of 2, and they had the same unidentified component as found in the thermolytic peptides.

Table 2. Enzymic activities of fragment A after ultraviolet-induced modification with NAD

	NAD-glycohydrolase		ADP-ribosyltransferase	
Fragment A	Specific activity*	% inactivation	Specific activity†	% inactivation
Nonirradiated	49.6	—	5.10	—
Irradiated, unlabeled	29.8	40	2.91	43
Irradiated, labeled	0.4	99	0.01	100

Fragment A and [*carbonyl*-^{14}C]NAD were mixed as described for Table 1 and irradiated at 0°C in 50 mM Tris·HCl, pH 7.2, for 30 min. After removal of reaction by-products by chromatography on Sephadex G-50, the sample was applied to a 2.0-ml column of NAD-agarose equilibrated in the same buffer. Irradiated radiolabeled material passing through the column was collected, and the irradiated, unlabeled fraction was eluted with 10 mM NAD. The labeled fraction contained 1.03 mol of ^{14}C per mol of fragment A. Both samples were dialyzed prior to a determination of enzymic activities.
*pmol of NAD hydrolyzed per 1.5 hr per 4 μg of protein.
†pmol of ADP-ribose incorporated per 15 min per 5 ng of protein.

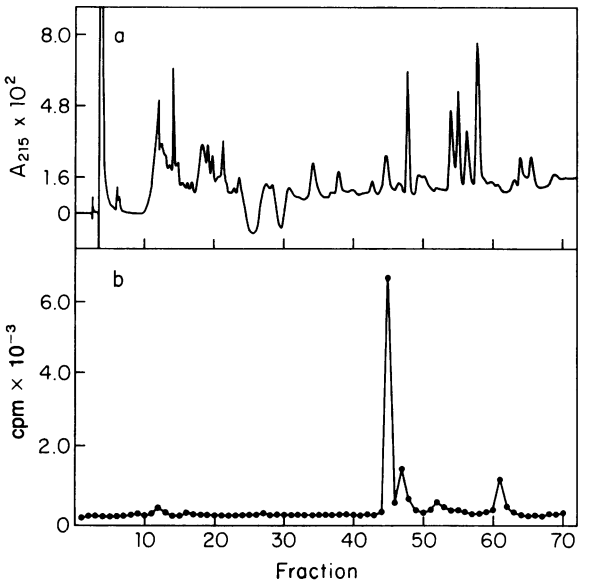

FIG. 3. HPLC elution profile of peptides derived from radiolabeled CNBr-3 by digestion with thermolysin. The major radiolabeled peptide peak obtained after cleavage with cyanogen bromide (CNBr-3, Fig. 2) was digested with 2% thermolysin in 0.2 M *N*-ethylmorpholine acetate, pH 8.2, for 4 hr at 45°C. The reaction was terminated by the addition of glacial acetic acid, and the sample was lyophilized. After resuspension in a minimal amount of 98% formic acid, the digest was diluted with 0.1% trifluoroacetic acid and chromatographed on an Alltech C_{18} reverse-phase column. Peptides were eluted with a linear 180-min gradient of 0–60% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid, with absorbance monitoring at 215 nm (*a*). One-milliliter fractions were collected, and 5-μl aliquots were assayed for radioactivity (*b*).

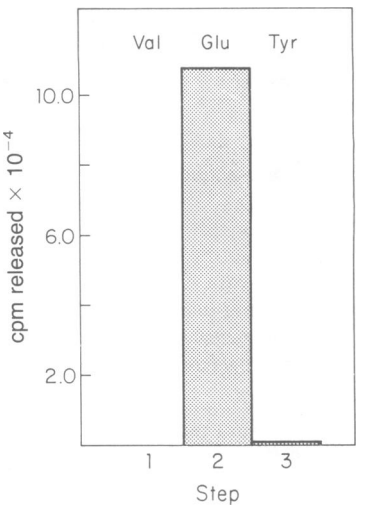

FIG. 4. Localization of the photoproduct within the thermolytic tripeptide from fragment A. The major radiolabeled thermolytic peptide (see Fig. 3) was sequenced by manual Edman degradation. Samples from each extraction step were assayed for radioactivity. The remaining extract was converted to the phenylthiohydantoin derivative by incubation in 1 M methanolic HCl and identified by thin-layer chromatography.

Digestion of the cyanogen bromide-resistant, higher molecular weight material (first peak of Fig. 2) with thermolysin or chymotrypsin yielded radiolabeled peptides identical to those described above. The failure of repeated cyanogen bromide treatment to alter the mobility of this material on Sephadex G-75 probably resulted from ultraviolet-induced intramolecular crosslinking or oxidation of methionine residue(s).

Position of the Radiolabeled Photoadduct. The major labeled thermolytic peptide was sequenced manually by the dansyl–Edman and Edman methods and found to be Val-X-Tyr. Virtually all (99%) of the radioactivity was released at the second position (X), corresponding to a derivative of Glu-148 (Fig. 4). The thermolytic peptide eluting at 47 min gave an identical NH_2-terminal sequence and position of isotopic release. Together, these two peptides accounted for greater than 87% of the incorporated radioactivity. Amounts of the 61 min peak were insufficient for further study.

Analysis of the labeled chymotryptic peptide also verified the sequence Ala-Glu-Gly-Ser-Ser-Ser-Val-X-Tyr, and most of the radioactive label (94%) was again released at position 148. Identical patterns of release were obtained with labeled peptides derived from either CNBr-3 or that material migrating with intact fragment A. As with the major thermolytic peptide, treatment of the chymotryptic peptide with carboxypeptidase A liberated only Tyr. Thus, at least 94% of the isotope incorporated during photolysis of nicotinamide-labeled NAD and DT fragment A was localized to a single residue, Glu-148.

Nature of the Photoproduct. Difference spectra between the fragment A-NAD photoproduct and fragment A irradiated in the absence of NAD showed a well-defined peak with a λ_{max} of *ca.* 260 nm, consistent with the notion that an aromatic moiety of NAD had been transferred to the protein. In addition, radiolabeled thermolytic or chymotrypic peptides showed an absorption peak at 264 nm, with an extinction coefficient of 6.1 mM^{-1} cm^{-1} at 260 nm. Since little absorption due to the tyrosine residue would be expected at this wavelength, these data suggested that the nicotinamide ring (or a derivative thereof) was covalently transferred to the A fragment. For convenience, we refer to this photoproduct as nicotinamide-labeled fragment A.

Boronate affinity gels failed to retain nicotinamide-labeled fragment A or its peptides, even after denaturation of the protein in 4 M urea. In contrast, radiolabeled peptides derived from fragment A photolyzed in the presence of phosphate- or adenine-labeled NAD bound quantitatively to such resins and could be recovered by elution at pH 5.0. Since boronate gels bind compounds containing unblocked vicinal hydroxyls, neither of the ribosyl moieties of NAD was apparently present in the nicotinamide-labeled photoproduct.

DISCUSSION

Prior studies from this laboratory and others showed that DT fragment A contains a single NAD site, with subsites for both aromatic moieties of the dinucleotide (6, 23). Subsequently we observed ultraviolet-induced linking between fragment A and NAD (13), presumably mediated by photoexcitation of either or both of the ligand's nitrogenous bases. The present results indicate that the entire NAD molecule does not become covalently associated with fragment A during photolysis. Rather, label contained within the nicotinamide moiety of NAD was transferred with high efficiency, whereas label within the AMP moiety was incorporated only poorly.

The optical properties of photolabeled fragment A and of peptides derived from it by proteolysis are consistent with the notion that it is the nicotinamide moiety that is efficiently linked to the protein. This would imply that the nicotinamide-ribose linkage of NAD is cleaved either during photolysis or subsequently by a chemical or enzymic mechanism. It has been reported (24, 25), and we have confirmed, that nicotinamide and ADP are the major products resulting from ultraviolet irradiation of NAD in solution.

We cannot exclude the possibility that the NMN phosphate or a derivative of the NMN ribose may be present in the carbonyl-^{14}C-labeled photoproduct, but the inability of

labeled peptides to bind boronate affinity gels indicates the absence of vicinal hydroxyl groups, and hence, of an intact ribose ring. Nuclear magnetic resonance and mass spectrometric studies should yield more definitive information concerning the nature of the transferred moiety.

The fact that most, if not all, of the label transferred from [carbonyl-^{14}C]NAD to fragment A during photolysis was attached to Glu-148 indicates that this residue is within the NAD binding site of DT. Additional evidence supporting this interpretation comes from studies with other forms of DT. Those forms that are known to have accessible NAD sites (fragment A, CRM-45, and the nucleotide-free form of whole toxin) were efficiently photolabeled in the presence of [*carbonyl*-^{14}C]NAD, whereas forms with altered or blocked NAD sites (CRM-197 and the nucleotide-bound form of whole toxin) exhibited greatly reduced levels of incorporation. Similarly, the NAD-binding site of exotoxin A is not exposed in the native toxin (26, 27), and photochemical labeling occurred only with the activated form.

It is noteworthy that Glu-148 is close in the primary structure to Trp-153, since earlier results suggested that the nicotinamide moiety of NAD interacted with a tryptophan residue of the A fragment. NAD binding to free fragment A (6) or the nucleotide-free form of DT (10) dramatically quenches intrinsic tryptophan fluorescence of the protein and creates a new absorbance peak at 360 nm. Both phenomena are dependent upon an N-substituted nicotinamide ring, and possibly result from a charge-transfer complex between the positively charged nicotinamide and an adjacent indole side chain (6). Trp-153 is one of only two tryptophan residues present in fragment A (the other occurring at position 50), and chemical modification of this residue is known to block enzymic activity (28).

If, as our data indicate, Glu-148 is contiguous with the nicotinamide moiety of bound NAD, then this residue can be no more than a few angstroms from the catalytic center of the toxin. The exceptional efficiency and specificity of photochemical labeling may reflect an interaction of Glu-148 in catalysis, but we have no direct evidence bearing on this point. Regardless, it seems likely that electrostatic interactions between the carboxyl anion of Glu-148 and the nicotinamide cation of NAD contribute to the binding of the dinucleotide to fragment A.

How general the phenomenon of efficient photolabeling by nicotinamide-labeled NAD is remains to be determined. Among the other proteins examined, only the activated form of exotoxin A from *P. aeruginosa* showed significant levels of incorporation. Such efficient labeling of exotoxin A is not surprising, in view of the fact that the NAD-binding and enzymic properties of activated exotoxin A are virtually identical to those of DT fragment A (29). Inasmuch as the NAD dissociation constants of the other proteins tested are much higher than those of the two toxins, a lower percentage of the binding sites would have been in liganded form under the conditions employed for irradiation. Experiments are necessary to determine whether efficient and specific photochemical modification by nicotinamide-labeled NAD is unique to diphtheria toxin and exotoxin A, is unique to the ADP-ribosylating exotoxins, or also occurs with other classes of NAD-binding proteins.

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