Purification of a Nitric Oxide-Stimulated ADP-Ribosylated Protein Using Biotinylated β-Nicotinamide Adenine Dinucleotide†

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ABSTRACT: ADP-ribosylation, involving the transfer of an ADP-ribose moiety from NAD to proteins, is mediated by several bacterial toxins and endogenous ADP-ribosyltransferases. We report here the synthesis of biotinylated NAD and its use to label and purify biotinyl-ADP-ribosylated proteins. We demonstrate that biotinylated NAD can be used by diphtheria toxin to biotinylate elongation factor 2. Using avidin affinity chromatography, we have purified a protein whose ADP-ribosylation is enhanced by nitric oxide and which has been identified as glyceraldehyde-3-phosphate dehydrogenase.

ADP-ribosylation of proteins catalyzed by toxins from bacteria, such as diphtheria, cholera, and pertussis, has been extensively studied (Ueda & Hayaishi, 1985). Much less is known of the role of ADP-ribosylation by endogenous, mammalian ADP-ribosyltransferases (Williamson & Moss, 1990). Recently Lapetina and associates (Brune & Lapetina, 1989) reported that nitric oxide (NO)[1] stimulates ADP-ribosylation of a protein in platelets. Because of the apparent importance of NO as a biological messenger in mediating the tumoricidal and bactericidal actions of macrophages (Nathan & Hibbs, 1991), dilating blood vessels (Moncada et al., 1991; Ignarro, 1990), and serving as a neurotransmitter in the central and peripheral nervous system (Bredt & Snyder, 1992), characterization of potential substrates for NO-stimulated ADP-ribosylation may be of importance. Many of the biological actions of NO involve stimulation of guanylyl cyclase (Schulz et al., 1991), and ADP-ribosylation may reflect an alternative target.

ADP-ribosylation is usually studied by utilizing [^{32}P]NAD as a substrate with subsequent characterization of the ^{32}P-labeled ADP-ribosylated protein. Because of the short half-life of ^{32}P as well as other technical factors, purification of ADP-ribosylated proteins may be difficult. In the present study we describe the synthesis of biotinylated NAD, which can be employed to yield biotinylated ADP-ribosylated proteins that can be purified by avidin affinity chromatography. We describe the purification to homogeneity of an NO-stimulated ADP-ribosylated protein (NAP) in rat brain.

EXPERIMENTAL PROCEDURES

Synthesis of Bio-NAD. NAD derivatives, which contain primary amine groups attached to positions N^6 or C^8 on adenine, were biotinylated by *N*-hydroxysuccinimide (NHS) derivatives of biotin that had spacer arms of different lengths. 8-[[*N*-biotinyl-(6-aminohexyl)]amino]NAD (8-bio-8-NAD) was synthesized from 8-[(6-aminohexyl)amino]NAD (Sigma) (20 mM in 0.1 M sodium borate, pH 8.5) and biotinyl *N*-hydroxysuccinimide (NHS-biotin) (Pierce) [0.2 M in dimethylformamide (Aldrich Chemical)]. N^6-[[[*N*-biotinyl-(6-aminohexyl)]carbamoyl]methyl]NAD (6-bio-10-NAD) was from N^6-[[(6-aminohexyl)carbamoyl]methyl]NAD (Sigma) and NHS-biotin. N^6-[[[*N*-[*N*-biotinyl-(ε-aminocapronyl)]-(6-aminohexyl)]carbamoyl]methyl]NAD (6-bio-17-NAD) was from N^6-[[(6-aminohexyl)carbamoyl]methyl]NAD and *N*-biotinyl-(ε-aminocaproic acid) *N*-hydroxysuccinimide (NHS-LC-biotin, Pierce). The amine derivatives of NAD and NHS derivatives of biotin were mixed in a 1:2 molar ratio. The reaction was kept at 25 °C in the dark for 48 h. The synthetic reaction of bio-NAD involves the primary amine derivative of NAD and an NHS derivative of biotin as precursors. *N*-Hydroxysuccinimide ester reacts with the primary amine to form an amide bond. The reaction occurs via a nucleophilic attack of an amine toward the NHS ester. This results in a stable amide bond and the release of NHS as a byproduct. The reaction is favored at alkaline pH, because the primary amine is kept in an unprotonated state. A secondary amine would not participate in the reaction under these conditions. According to the manufacturer (Pierce Bulletin 21336, p 2), a molar ratio of 1:22 (NHS-LC-biotin/monoclonal antibodies) yields biotin substitution levels of 8–14 M NHS-LC-biotin/1 M monoclonal antibody. The 1:2 molar ratio we employed was expected to yield less than 1 M biotin/1 M NAD. This condition ensures the addition of biotin to the most reactive primary amine group on NAD derivatives. Unbiotinylated NAD precursor was subsequently separated by HPLC.

Purification of Bio-NAD. Bio-NAD was purified by reverse-phase chromatography on C_{18} columns. On an analytic scale, 10 μL of reaction mixture was diluted to 100 μL with water and loaded on a C_{18} column (2.1 × 220 mm, Aquapore OD-300, 7 μm, Applied Biosystems). A linear gradient with 25 mM ammonium bicarbonate (NH_4HCO_3) (pH 7.7) as buffer A and 70% (v/v) methanol as buffer B was applied at a rate of 1 mL/min while absorbance was monitored at 260 nm. The gradient started at 0% buffer B at 0 min, increased to 37.5% B at 15 min, and decreased to 0% B at 18 min. We first calibrated the column with individual standard precursors

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[1] Abbreviations: NAD, β-nicotinamide adenine dinucleotide; 6-bio-10-NAD, N^6-[[[*N*-biotinyl-(6-aminohexyl)]carbamoyl]methyl]NAD; 6-bio-17-NAD,N^6-[[[*N*-[*N*-biotinyl-(ε-aminocapronyl)]-(6-aminohexyl)]-carbamoyl]methyl]NAD; 8-bio-8-NAD, 8-[[*N*-biotinyl-(6-aminohexyl)]-amino]NAD; bio-NAD, biotinylated NAD; DTT, dithiothreitol; EF-2, elongation factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; NAP, nitric oxide-enhanced ADP-ribosylated protein; NHS, *N*-hydroxysuccinimide; NO, nitric oxide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNP, sodium nitroprusside.

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of the biotinylation reaction, NHS-LC-biotin, NHS-biotin, 8-[[*N*-biotinyl-(6-aminohexyl)]amino]NAD, N^6-[[(6-aminohexyl)carbamoyl]methyl]NAD, and one product of the reaction, NHS. The only other product of the reaction should be bio-NAD, which eluted at a position different from the reactants and byproduct. The elution points for 8-bio-8-NAD, 6-bio-10-NAD, and 6-bio-17-NAD were 9.7, 8.2, and 10.9 min, respectively. Bio-NAD was collected and concentrated by speed vacuum drying. On a preparative scale, 200 μL of reaction mixture was loaded on a C_{18} column (4.6 × 250 mm, 10 μm, Alltech/Applied Science). The same gradient as for analytic scale was used except that the flow rate was increased to 2 mL/min. Chromatographic profiles for analytic and preparative purifications showed essentially the same pattern.

Determination of Bio-NAD Concentration. Since bio-NAD has the same pattern of UV spectrum as its precursor, (i.e., the spectra for 8-bio-8-NAD and 8-[(6-aminohexyl)amino]-NAD are superimposable, and the same for 6-bio-10-NAD or 6-bio-17-NAD and N^6-[[(6-aminohexyl)carbamoyl]methyl]NAD), we assume that bio-NAD has the same extinction coefficient as its precursor. Therefore we used the extinction coefficients of 19 000 M^{-1} cm^{-1} (280 nm) for 8-[(6-aminohexyl)amino]NAD and 22 000 M^{-1} cm^{-1} (265 nm) for N^6-[[(6-aminohexyl)carbamoyl]methyl]NAD (Schmidt & Dolabdjian, 1980) to calculate the concentrations for 8-bio-8-NAD and 6-bio-10-NAD/6-bio-17-NAD, respectively.

Preparation of Brain Extract. Adult rat brain was homogenized in 8× (w/v) ice-cold buffer consisting of 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 1 μg/mL each chymostatin, leupeptin, pepstatin, and trypsin inhibitor (all from Sigma). The homogenate was centrifuged at 100000*g* for 40 min. The supernatant was used for in vitro ADP-ribosylation.

In Vitro ADP-Ribosylation of NAP. A typical ADP-ribosylation reaction contained 10 μL of brain extract and freshly prepared solution containing 0.5 mM sodium nitroprusside (SNP) (Sigma) and 0.5 μM [*adenylate*-^{32}P]NAD (800 Ci/mmol, NEN/DuPont) or 60 μM bio-NAD to a final volume of 20 μL. For competition assays, competitors (either nonradioactive NAD, 8-bio-8-NAD, 6-bio-10-NAD, or 6-bio-17-NAD) at concentrations of 2.5, 25, or 250 μM were simultaneously added into the ^{32}P-labeling mixture containing 0.5 μM [^{32}P]NAD. For NAP purification, 6-bio-17-NAD was used to label a 20-mL reaction mixture. All the reactions were incubated at 37 °C for 1 h. In one experiment, the ^{32}P-labeled mixture was treated, at the end of 1 h of labeling, with either 5 mM mercuric chloride or 200 mM hydroxylamine (pH 7.4) for another 0.5 h at 37 °C. All ^{32}P-labeling reactions were terminated with 20% trichloroacetic acid. The pellet was washed with acetone and then solubilized in SDS gel-loading buffer. The biotinylation reaction was terminated with 2× SDS gel-loading buffer. Proteins were resolved on a sodium dodecyl sulfate–12.5% polyacrylamide gel and detected as described below.

ADP-Ribosylation of Elongation Factor 2. EF-2 (0.1 μg), purified from 3T3 L1 cells by Dr. K. Liao (Department of Biological Chemistry, Johns Hopkins University School of Medicine), was incubated with 1 ng of diphtheria toxin (Calbiochem) in 25 μL of 20 mM Tris-HCl (pH 7.4)/1 mM EDTA/1 mM DTT, either without NAD or with 5 μM [^{32}P]-NAD or 50 μM 8-bio-8-NAD, 6-bio-10-NAD, or 6-bio-17-NAD. The reaction mixture was kept at 37 °C for 1 h and terminated with 2× SDS gel-loading buffer. Proteins were separated on an SDS–10% polyacrylamide gel. ADP-ribosylated EF-2 was detected as described below.

Detection of ADP-Ribosylated NAD or EF-2. ^{32}P-labeled NAP or EF-2 was detected by autoradiography after electrophoresis on SDS–12.5% or 10% polyacrylamide gels. To detect biotinylated NAP or EF-2, proteins were electrophoretically transferred from SDS gels to nitrocellulose. The nitrocellulose filter was blotted with 0.5 μg/mL horseradish peroxidase (HRP)–avidin D (Vector Laboratories) and 2% bovine serum albumin in buffer C consisting of 10 mM sodium phosphate (pH 7.4), 140 mM NaCl, and 0.5% Triton X-100 for 1 h, followed by 4 × 10 min washing with buffer C, and developed by the enhanced chemiluminescence (ECL) method according to the manufacturer's protocol (Amersham).

Purification of NAP. All the purification steps were carried out at 4 °C except where indicated. After in vitro biotinyl-ADP-ribosylation with 6-bio-17-NAD, proteins were first fractionated by ammonium sulfate [$(NH_4)_2SO_4$] precipitation. The reaction mixture was adjusted to 65% $(NH_4)_2SO_4$ with saturated $(NH_4)_2SO_4$ solution and then centrifuged at 13000*g* for 15 min to remove the pellet. The NAP-containing supernatant (25 mL) was chromatographed by fast protein liquid chromatography on an HR5/5 phenyl-Superose column (Pharmacia) equilibrated with 2.7 M $(NH_4)_2SO_4$ in buffer D [25 mM Tris-HCl (pH 7.4)/1 mM EDTA]. A two-step gradient was conducted. The initial linear gradient was increased from 0 to 50% D over 5 mL and was followed by a second gradient to 80% D over 30 mL. The phenyl-Superose NAP fraction was dialyzed against 0.1 M NH_4HCO_3 (pH 7.7) and further purified by HR10/30 Superose-12 gel filtration. For each Superose-12 chromatograph, an 0.5-mL phenyl-Superose NAP fraction was loaded and eluted with 0.1 M NH_4HCO_3 (pH 7.7). The Superose-12 NAP fraction (20 mL) was loaded onto a 1-mL agarose avidin D column (Vector Laboratories). After washes with 20 mL of 25 mM Tris-HCl (pH 7.4)/1 mM EDTA/0.5 M NaCl and then 5 mL of 25 mM Tris-HCl (pH 7.4)/1 mM EDTA, NAP was eluted by 5 mM mercuric chloride at 25 °C.

RESULTS

Synthesis of Biotinylated NAD and Its Use as a Substrate in ADP-Ribosylation of Elongation Factor 2. The available modification sites on NAD where biotin might be added for ADP-ribosylation would be either on adenosine or on ribose but not on nicotinamide. Biotinylation on ribose through periodate oxidation and biotin hydrazide condensation would disrupt the two ribose rings and introduce four biotins per NAD. This would change the structure of NAD too much to permit ADP-ribosylation. We therefore chose to biotinylate on the adenosine group in NAD. In case some modification on NAD rendered it unusable by certain ADP-ribosyltransferases, we synthesized several derivatives of NAD biotinylated on either the N^6 or C^8 positions of the adenine (Figure 1). One of these, designated 8-bio-8-NAD, utilizes a spacer group incorporating eight carbons between the adenine and the biotin with the biotin attached to the 8-position of adenine. In the numbering system employed, the first number refers to the position on adenine to which the spacer link is added, while the second number refers to the number of equivalent carbons separating adenine from biotin. We found that the length of the spacer arm was important to overcome steric hindrance for binding of biotin to avidin, especially when the ADP-ribosylation sites were buried inside the proteins. Two other derivatives were synthesized with 10- or 17-carbon spacers between the 6-position of adenine and biotin, designated 6-bio-10-NAD and 6-bio-17-NAD, respectively.

For the synthesis of 8-bio-8-NAD, 8-[(6-aminohexyl)amino]-NAD was reacted with NHS-biotin (Figure 1). Precursor

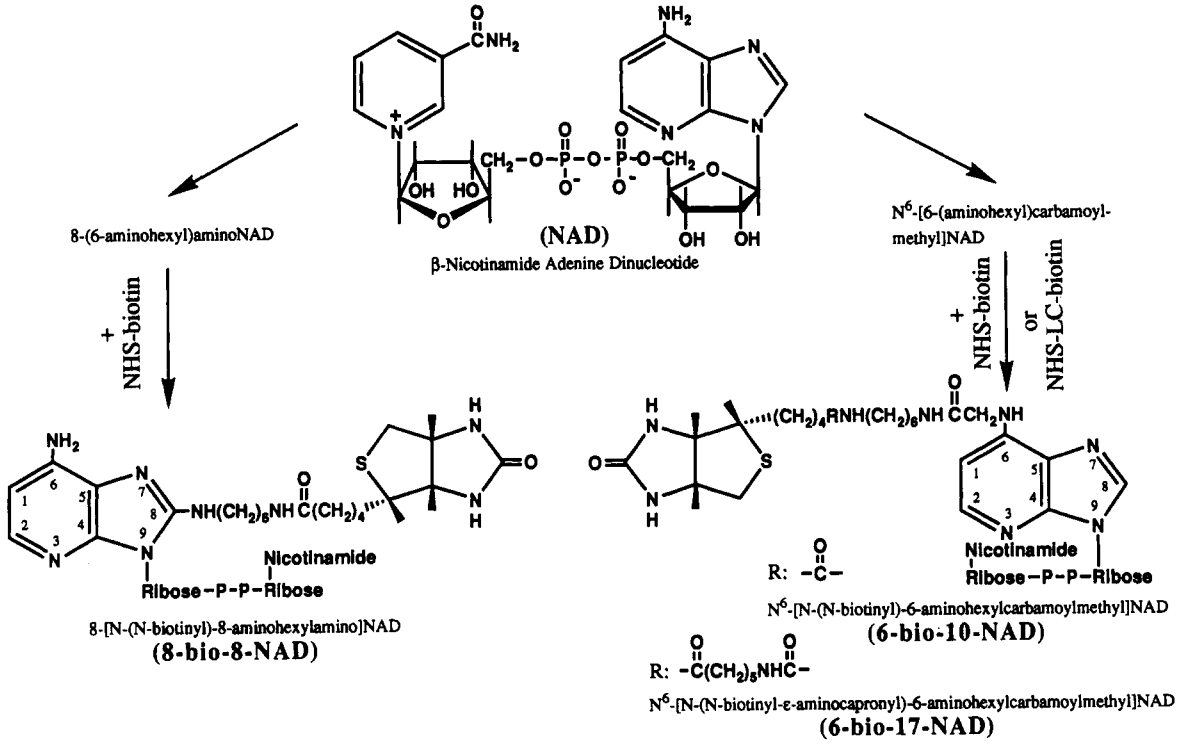

FIGURE 1: Scheme for synthesis of bio-NAD. Pathways for chemical synthesis of 8-bio-8-NAD, 6-bio-10-NAD, and 6-bio-17-NAD are illustrated. See Experimental Procedures for details.

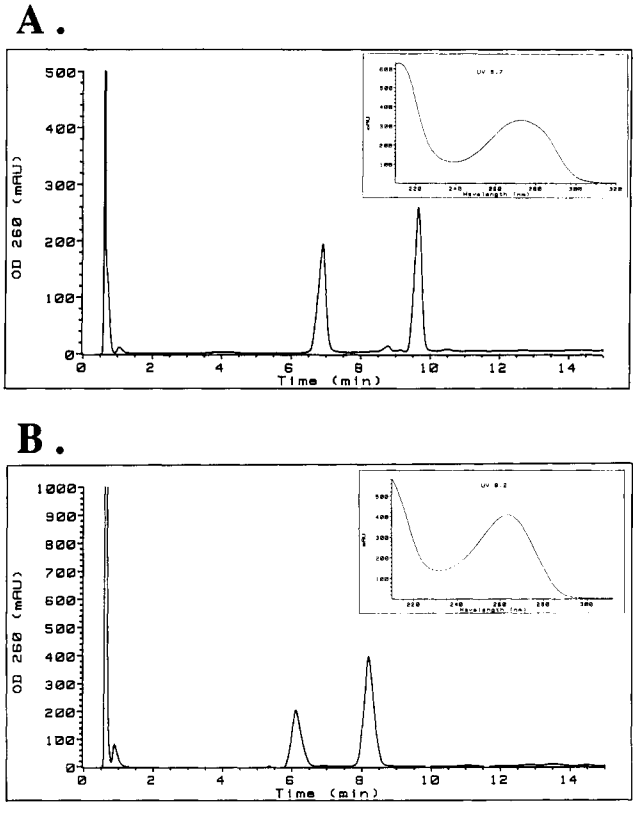

FIGURE 2: HPLC purification of bio-NAD. 8-Bio-8-NAD (panel A) and 6-bio-8-NAD (panel B) were purified by C_{18} reverse-phase chromatography. Shown here are the profiles with absorbance at 260 nm as a function of elution time. Spectra of bio-NAD are shown in the insets. See Experimental Procedures for detailed procedures of bio-NAD purification.

and product were separated by reverse-phase HPLC (Figure 2A). The precursor emerged before the product, which eluted at 9.7 min. Its ultraviolet (UV) spectrum, with a peak at 280 nm characteristic of 8-[(6-aminohexyl)amino]NAD derivative (Lee et al., 1974), provided a means of estimating the concentration of 8-bio-8-NAD. No side reaction on N^6 was observed, as no peak was detected at 8.2 min, where 6-bio-10-NAD was eluted. 6-Bio-10-NAD was synthesized by reacting N^6-[[(6-aminohexyl)carbamoyl]methyl]NAD with NHS-biotin. 6-Bio-17-NAD utilized the same NAD derivative reacted with NHS-LC-biotin. In reverse-phase HPLC the precursor emerged before the product with 6-bio-10-NAD emerging at 8.2 min. Its UV spectrum, with a peak at 265 nm, was characteristic of the synthetic precursor (Lindberg et al., 1973). The HPLC elution profile for 6-bio-10-NAD (Figure 2B) was essentially the same as for 6-bio-17-NAD (data not shown).

The presence of biotin in the synthesized biotinylated NAD molecules was confirmed by dot blots in which these substances, applied to nitrocellulose, were reacted with avidin-conjugated horseradish peroxidase (HRP). Moreover, avidin–agarose beads precipitated these substances, as monitored by UV absorbance, from solution. This ensures the expected property and purity of bio-NAD.

To ascertain whether the biotinylated forms of NAD can serve as substrates for ADP-ribosylation, we tested their ability to subserve ADP-ribosylation of elongation factor 2 (EF-2) catalyzed by diphtheria toxin (Figure 3). Utilizing [^{32}P]NAD and monitoring radioactive product by autoradiography following SDS–PAGE analysis, a single 100-kDa band, corresponding to the size of EF-2 (Riis et al., 1990), was labeled. Utilizing the biotinylated forms of NAD, the ADP-ribosylated product was detected by avidin-conjugated HRP coupled to the ECL detection system. With 8-bio-8-NAD, 6-bio-10-NDA, and 6-bio-17-NAD, 100-kDa bands were observed. Thus, the three forms of bio-NAD can serve as substrates for ADP-ribosylation. The faint band at about 70 kDa in lanes 2–4 was not protein since it failed to stain with Coomassie Blue. The absence of a 70-kDa band in lane 1 suggested that the band could be residual bio-NAD retained

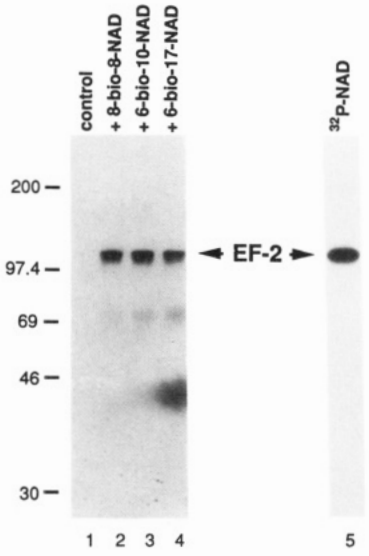

FIGURE 3: Biotinylation of elongation factor 2. Purified EF-2 was incubated with diphtheria toxin in the absence of NAD (lane 1) or in the presence of 8-bio-8-NAD (lane 2), 6-bio-10-NAD (lane 3), or 6-bio-17-NAD (lane 4). Proteins were resolved by SDS–10% PAGE and electrophoretically transferred to nitrocellulose. HRP-conjugated avidin coupled to ECL detects the biotinyl-ADP-ribosylated EF-2, which migrates at the same position as [^{32}P]ADP-ribosyl-EF-2 on the autoradiogram (lane 5). The standard molecular masses in kilodaltons and the position for EF-2 are indicated.

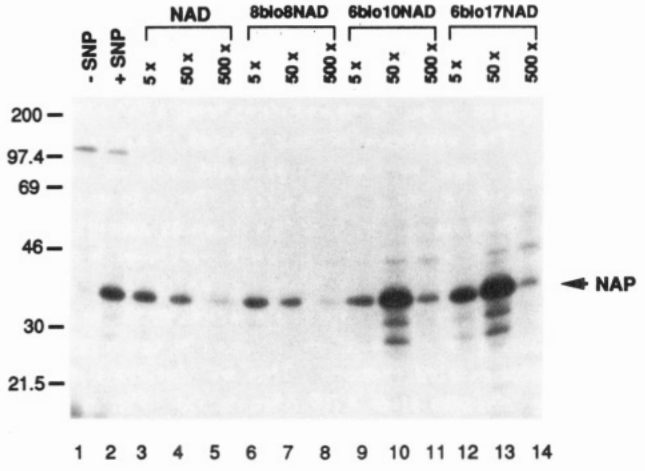

FIGURE 4: Bio-NAD competes with [^{32}P]NAD as donor of ADP-ribose for in vitro ADP-ribosylation. An autoradiogram of NO-enhanced ADP-ribosylation of 37-kDa protein with [^{32}P]NAD is shown. In the presence of NO, generated from SNP (lane 2), ADP-ribosylation of NAP increases 15-fold (compare with lane 1). This ^{32}P-labeled signal can be competitively diminished with increasing concentrations of nonradioactive NAD (lanes 3–5), 8-bio-8-NAD (lanes 6–8), 6-bio-10-NAD (lanes 9–11), or 6-bio-17-NAD (lanes 12–14). The standard molecular masses in kilodaltons are indicated on the left and the position for NAP at right with an arrow.

on nitrocellulose. 6-Bio-17-NAD and 8-bio-8-NAD, when running alone on SDS gels, could be detected at the 70-kDa region after the electrophoretic transferring time was shortened (data not shown). The smear band of 45 kDa in lane 4 appeared to be an artifact of ECL and was not reproducible.

Identification of NO-Stimulated ADP-Ribosylation of a Protein in Rat Brain Utilizing Biotinylated NAD. In platelets, Lapetina and associates (Brune & Lapetina, 1989) reported that sodium nitroprusside (SNP), which generates NO, enhances ADP-ribosylation of a 39-kDa protein. In rat brain extracts utilizing [^{32}P]NAD, SNP markedly stimulated labeling of a 37-kDa band (Figure 4). To ascertain whether the biotinylated NAD compounds interact with the same site

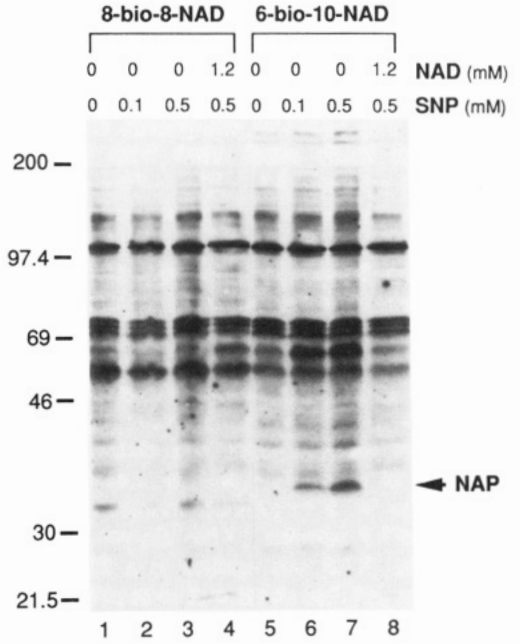

FIGURE 5: Bio-NAD biotinylates substrate proteins of ADP-ribosylation. 8-Bio-8-NAD and 6-bio-10-NAD were used for *in vitro* ADP-ribosylation of NAP. After SDS–12.5% PAGE and electrophoretic transfer of proteins to nitrocellulose, the biotinyl-ADP-ribosylated NAP was detected by HRP–avidin coupled to an ECL system. As indicated by the arrow, ADP-ribosylation of NAP with 6-bio-10-NAD is stimulated with increasing concentrations of SNP (lanes 6 and 7). NAD can competitively abolish the biotin labeling (lane 8), demonstrating the specificity of ADP-ribosylation. The HRP/ECL system fails to detect NAP labeled with 8-bio-8-NAD (lanes 1–4), perhaps due to steric hindrance. Molecular masses in kilodaltons are indicated at left and the position for NAP at right with an arrow.

as NAD, we evaluated their ability to compete for labeling of the NO-enhanced ADP-ribosylated protein (NAP). At concentrations 5, 50, and 500 times that of [^{32}P]NAD (2.5 μM), NAD progressively decreased labeling of NAP. 8-Bio-8-NAD provided a similar concentration-dependent reduction of labeling. With 6-bio-10-NAD and 6-bio-17-NAD, the 50-fold increased concentration actually enhanced NAP labeling, while at 500× markedly diminished labeling was observed. Though the exact mechanism for this effect is unclear, conceivably 6-bio-10-NAD and 6-bio-17-NAD bind to multiple ADP-ribosylation sites which cooperatively stimulate [^{32}P]ADP-ribose incorporation into NAP. Such cooperativity might derive from the tetrameric state of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which we found to represent NAP (Zhang & Snyder, 1992). We reasoned that the biotin moiety on the 6-position of bio-NAD would change the conformation of GAPDH such that the remaining NAD sites were more readily occupied by [^{32}P]NAD.

We examined the direct incorporation of the various forms of bio-NAD into NAP (Figure 5). Substantial SNP-stimulated labeling of NAP was observed for 6-bio-10-NAD (Figure 5) and for 6-bio-17-NAD (data not shown). However, NAP labeled with 6-bio-10-NAD could not be precipitated by avidin–agarose, while NAP labeled with 6-bio-17-NAD was precipitated (see below). This suggested that the ADP-ribosylation site might be buried inside the protein so that when NAP was labeled with bio-NAD containing a short spacer arm, such as 6-bio-10-NAD, it had to be denatured to expose biotin for detection by avidin-HRP. Furthermore, 8-bio-8-NAD failed to provide labeling of NAP, which might again reflect steric hindrance between biotin and avidin, due to a short spacer arm (Figure 5). A direct test of this possibility

was hindered by the unavailability of C^8 primary amine derivatives of NAD to synthesize 8-bio-NAD with a spacer arm as long as that of 6-bio-17-NAD. A number of other labeled bands did not show enhanced labeling with SNP. Some of these bands may represent endogenous biotin-containing proteins. Other bands could result from the nonspecific interaction with avidin–HRP in ECL. They were present in the whole brain extract even without incubation with bio-NAD (data not shown). These bands were not labeled through ADP-ribosylation, because the "labeling" could not be removed by treatment with phosphodiesterase (data not shown).

Purification of NAP. In initial experiments we observed that NAP is quite soluble in ammonium sulfate. It remains soluble in 65% ammonium sulfate. Thus, the supernatant from 65% ammonium sulfate treatment was applied to a phenyl-Superose column which was eluted by decreasing concentrations of ammonium sulfate. Individual fractions were assayed for NAP by an ECL detection system for the biotin/avidin–HRP complex. After dialysis to remove ammonium sulfate, NAP was applied to a Superose-12 gel-filtration column and then eluted with ammonium bicarbonate. NAP migrated at about the same position as γ-globulin, whose molecular weight is 158 000, indicating that NAP probably is a tetramer (data not shown).

The final purification step involved affinity chromatography with avidin–agarose. Although NAP modified by 6-bio-10-NAD was easily detected on nitrocellulose when it was denatured (Figure 5), it bound very poorly to avidin–agarose in its native state. This suggested that the length of the spacer arm was important to overcome the steric hindrance of avidin/biotin binding. When we employed 6-bio-17-NAD to label NAP, binding between 6-bio-17-ADP-ribosyl-NAP and agarose increased significantly. Because of the extremely high affinity of biotin for avidin, it is difficult to elute such columns. ADP-ribosylation often involves arginine or lysine in interactions that can be disrupted by hydroxylamine (Hilz et al., 1982) or cysteine whose interactions can be reversed by mercuric chloride (Aktories et al., 1988). To identify the amino acid residue in NAP that is ADP-ribosylated, we first labeled NAP in rat brain homogenates with [^{32}P]NAD (Figure 6, lane 1). Then we added mercuric chloride or hydroxylamine to the mixture (Figure 6, lanes 2 and 3). Hydroxylamine failed to decrease labeling of NAP, while mercuric chloride abolished such labeling. The enhanced labeling in the presence of hydroxylamine presumably reflects its release of NO (Southam & Garthwaite, 1991). Although the exact mechanism of NO release from hydroxylamine is unknown, this oxygen-dependent conversion could be catalyzed by catalase and presumably other metalloproteins containing heme or flavin moieties which are involved in oxidation–reduction reactions (Waldman & Murad, 1987).

Because of the ability of mercuric chloride to disrupt NAP labeling, we employed it to elute the avidin affinity column. This chemical elimination process enabled us to purify NAD to homogeneity using the avidin column (data not shown).

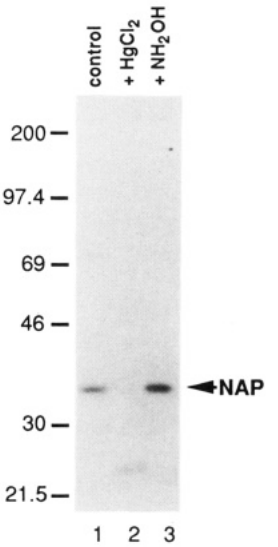

FIGURE 6: Differential removal of ADP-ribosyl group from NAP. [^{32}P]NAD was used as the ADP-ribose donor for in vitro ADP-ribosylation of rat brain extract. The initial ^{32}P-label on NAP (lane 1) can subsequently be abolished by further incubating the ADP-ribosylation mixture with mercuric chloride (lane 2) but not hydroxylamine (lane 3). Molecular masses in kilodaltons are indicated at left and the position for NAP at right with an arrow.

DISCUSSION

ADP-ribosylation was first discovered as a reaction catalyzed by bacterial toxins labeling a few mammalian substrates such as EF-2 and G proteins (Ueda & Hayaishi, 1985). Subsequently it became apparent that endogenous ADP-ribosyltransferases exist so that ADP-ribosylation may be a physiological process (Williamson & Moss, 1990). Numerous protein substrates have been identified, suggesting that ADP-ribosylation is a major regulator of protein function, comparable to phosphorylation (Moss & Vaughan, 1988). However, outside of G proteins, few substrates of ADP-ribosylation in mammalian systems have been isolated and identified.

Our system utilizing biotinylated NAD may afford an efficient means for purifying and identifying ADP-ribosylated proteins. The synthesis of biotinylated NAD is simple, involving a single step with high yield, and no extensive purification of the product is required. The technique is quite sensitive. Because biotinylated NAD can be separated from NAD readily, there is no dilution of biotinylated NAD with NAD, in contrast to [^{32}P]NAD in which only a small proportion of the NAD molecules are radiolabeled. In contrast to ^{32}P-labeling, biotinylated proteins can be easily separated from unbiotinylated proteins. This will facilitate study of functional changes after ADP-ribosylation. The sensitivity for detection of biotinylated ADP-ribosylated proteins is very high. This depends in part upon the amplification afforded by the peroxidase enzymatic reaction. Additionally, the ECL detection system is sensitive to picogram levels. Sensitivity is also enhanced by the lack of a requirement for a secondary antibody and by the high-affinity binding of avidin to biotin.

We demonstrated the application of biotinylated NAD for labeling two ADP-ribosylated proteins, EF-2 and NAP. This suggests that the technique can be applied generally to most ADP-ribosylated proteins.

The technique can be modified for dealing with different proteins. NAP was eluted from the avidin affinity column with mercuric chloride. Proteins ADP-ribosylated on arginine may be eluted with hydroxylamine. One can separate proteins labeled on cysteine from those labeled on basic amino acids by differential elution with mercuric chloride and hydroxylamine. The spacer arm can be modified to incorporate disulfide bridges so that such columns could be eluted with DTT (Shimkus et al., 1985). To lessen the extremely tight binding of avidin to biotin, one could incorporate modified forms of biotin (Hofmann et al., 1982).

In other studies we (Zhang & Snyder, 1992) and others (Kots et al., 1992; Dimmeler et al., 1992) have shown that NAP represents glyceraldehyde-3-phosphate dehydrogenase

(GAPDH). GAPDH appears to be auto-ADP-ribosylated, as purified GAPDH is ADP-ribosylated in the presence of SNP without addition of any tissue extract (Zhang & Snyder, 1992; Dimmeler et al., 1992). Endogenous NO in the brain evokes ADP-ribosylation of GAPDH, as treatment of brain extracts with inhibitors of NO synthase decreases GAPDH ADP-ribosylation that is observed in the absence of SNP (Zhang & Snyder, 1992). Since GAPDH is ADP-ribosylated on the cysteine which is at the active site of the enzyme (Bode et al., 1975), this modification abolishes enzyme activity (Dimmeler et al., 1992). Thus, ADP-ribosylation of GAPDH appears to be a physiologic target for NO. The use of biotinylated NAD may facilitate more extensive screening of mammalian tissues for other instances of physiologically important ADP-ribosylation.

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