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Stimulation by nitric oxide of an NAD linkage to glyceraldehyde-3-phosphate dehydrogenase

(ADP-ribosylation/sodium nitroprusside/mercury)

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ABSTRACT **Nitric oxide-stimulated modification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by [*adenylate*-^{32}P]NAD has been interpreted in recent reports as ADP-ribosylation. Incubations of GAPDH with the NO-releasing agent sodium nitroprusside (SNP) and NAD resulted, however, in essentially equal incorporation of radiolabel from the adenine, phosphate, and nicotinamide moieties to the extent of ≈0.02 mol of NAD·mol of GAPDH^{-1}. Modification of GAPDH by free adenosine 5′-diphosphoribose (ADP-ribose) was only 10% of that by NAD. Exposure of GAPDH modified by NAD in the presence of SNP to $HgCl_2$, which acts at thiol linkages, released two products. Both contained nicotinamide and adenylate but did not cochromatograph with NAD. GAPDH activity was inhibited by SNP in a dose-dependent manner in the presence of NAD. When inhibition was 80%, with 1 mM SNP and 1 mM dithiothreitol, covalent modification with NAD was <2%. This result is consistent with the conclusion that inhibition of GAPDH activity by SNP in the presence of NAD is due primarily to active-site nitrosylation, as reported by other workers, and is not due to the minor modification with NAD. These results demonstrate that NO-stimulated modification of GAPDH with NAD is not ADP-ribosylation as previously reported but rather is covalent binding of NAD through a NO-dependent thiol intermediate, possibly providing an example of an unexpected, altered reactivity of a nitrosylated protein.**

Nitric oxide (NO) is a cellular mediator with multiple biological functions including smooth muscle relaxation, macrophage-mediated cytotoxicity, and neurotransmission (1). The intracellular targets regulated by NO are not fully characterized but include the specific activation of guanylate cyclase due to NO coordination to the enzyme heme and nonspecific effects on protein sulfhydryl groups and iron–sulfur centers (1, 2).

ADP-ribosylation is a regulatory protein modification in which the adenosine 5′-diphosphoribose (ADP-ribose) moiety of NAD is transferred from NAD to protein, catalyzed by a family of amino acid-specific ADP-ribosyltransferases, including both bacterial toxins and endogenous cellular enzymes (3, 4). Recent studies in permeabilized cells or in subcellular fractions demonstrated a NO-stimulated labeling by [*adenylate*-^{32}P]NAD of a 37- to 41-kDa protein (5–8). The modification was interpreted as an ADP-ribosylation resulting from stimulation by NO of an endogenous ADP-ribosyltransferase (5–8). After purification and microsequencing of the radiolabeled protein from human erythrocytes or platelets or rat brain, it was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) (9–12). Incorporation of radiolabel into isolated GAPDH was also stimulated by NO, meaning that an ADP-ribosyltransferase was not needed and suggesting that the protein was automodified (9–13). Label was released from GAPDH by treatment with mercuric ion, consistent with the presence of an ADP-ribosylcysteine linkage (9–13). The active-site cysteine of GAPDH was suggested to be the site of modification, so that ADP-ribosylation could account for the observed loss of GAPDH activity, even though the extent of modification was very low (≈1%) (9–13).

We have now further characterized the GAPDH modification. On the basis of radiolabeling, both the adenine and nicotinamide moieties of NAD were incorporated into the enzyme and released by treatment with Hg^{2+}, indicating that GAPDH was modified covalently with NAD, not with ADP-ribose.

MATERIALS AND METHODS

SDS/PAGE Analysis of Modified GAPDH. Rabbit muscle GAPDH was supplied as a 10 mg·ml^{-1} suspension in 3.2 M $(NH_4)_2SO_4$ (Boehringer Mannheim). For use in labeling studies, a sample was centrifuged (14,000 × *g*, 10 min), the supernatant was removed, and the pellet was suspended at 5 mg·ml^{-1} in 0.5 M sodium-Mops (3-morpholinopropanesulfonic acid), pH 7.5/5 mM EDTA. GAPDH (40 μg; 11 μM monomer) was incubated with 0.1 mM of either [*nicotinamide*-^{14}C]NAD (Amersham; 57 mCi·mmol^{-1}; 1 Ci = 37 GBq), [*adenine*-^{14}C]NAD (Amersham; supplied as 275 mCi·mmol^{-1} and diluted with unlabeled NAD to 57 mCi·mmol^{-1}), [*adenylate*-^{32}P]NAD (NEN; 30 Ci·mmol^{-1}), or [*adenylate*-^{32}P]ADP-ribose, with or without 1 mM sodium nitroprusside (SNP) (Fisher) in 0.1 M sodium-Mops, pH 7.5/10 mM dithiothreitol/1 mM EDTA (total vol, 0.1 ml). Radiolabeled ADP-ribose was prepared from [^{32}P]NAD, as described (14), and both the ADP-ribose and NAD were purified by HPLC just before use. Unlabeled NAD and ADP-ribose were from Sigma. Reactions were incubated for 60 min at 30°C; then protein was precipitated with the addition of 1 ml of cold 10% trichloroacetic acid and incubation at 4°C for 30 min and was collected after centrifugation (10,000 × *g*, 20 min) and aspiration of the supernatant. The pellet was suspended in SDS/PAGE sample buffer and heated in boiling water for 5 min. Samples were subjected to electrophoresis in 12% acrylamide gels, along with prestained molecular weight markers (BRL). For autoradiography, gels containing ^{14}C-labeled GAPDH were fixed for 20 min in 10% acetic acid/20% methanol, treated with Pro-Mote (Integrated Separation Systems, Natick, MA), and dried under vacuum; x-ray film was exposed to the gels for 30 days. Gels with ^{32}P-labeled GAPDH were stained with Coomassie blue and dried; x-ray film was exposed to the gels for 16 hr. For analysis with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA),

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SNP, sodium nitroprusside.

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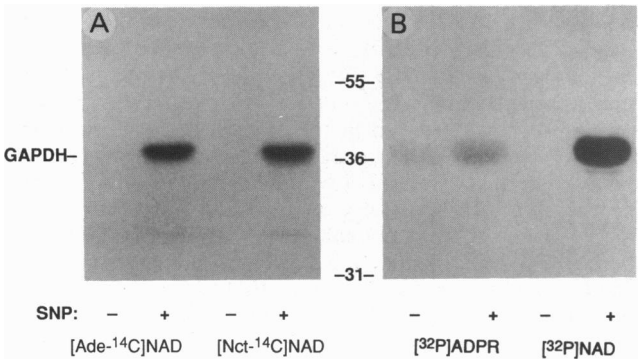

FIG. 1. Nitroprusside-stimulated incorporation of radioactivity from NAD into GAPDH. GAPDH was incubated with NAD or ADP-ribose and then analyzed by SDS/PAGE and autoradiography. (*A*) Incubation of GAPDH with [*adenine*-^{14}C]NAD or [*nicotinamide*-^{14}C]NAD ([Nct-^{14}C]NAD) without or with SNP. (*B*) Incubation of GAPDH with [*adenylate*-^{32}P]ADP-ribose ([^{32}P]ADPR) or [*adenylate*-^{32}P]NAD without or with SNP. Autoradiograms represent three independent experiments.

gels with ^{14}C-labeled GAPDH were processed as above but were not treated with Pro-Mote.

Determination of the Stoichiometry of GAPDH Modification. GAPDH was incubated with radiolabeled NAD in reactions containing GAPDH at 1 mg·ml^{-1} (28 μM GAPDH monomer), 0.25 mM total NAD, including either 0.65 μCi of [*nicotinamide*-^{14}C]NAD, 0.65 μCi of [*adenine*-^{14}C]NAD, or 1.7 μCi of [^{32}P]NAD, 100 mM sodium Mops, 10 mM dithiothreitol, and 1 mM EDTA with or without 1 mM SNP for 60 min at 30°C (total vol, 0.1 ml). Labeled GAPDH was separated from unbound NAD on PD-10 columns (Pharmacia) in 20 mM sodium Mops (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.01% Triton X-100. The early protein peak was collected (V_e = 2.75–3.5 ml); this fraction contained ≈70% of the applied protein and <0.001% of the applied free NAD. Protein (by the BCA assay, Pierce) and radioactivity (by liquid scintillation counting) were measured in this fraction. To determine NAD bound to denatured GAPDH, a sample from the PD-10 void fraction was made 1% in SDS and heated in boiling water for 5 min. Protein was precipitated with trichloroacetic acid, as described above and suspended in 1% SDS/100 mM sodium acetate (pH 6), 1 mM EDTA; samples were taken for protein assay and determination of radioactivity.

GAPDH Assay. The activity of GAPDH was determined by measuring the absorbance at 340 nm of NADH, a reaction product (15). Glyceraldehyde 3-phosphate (diethylacetal, barium salt; Boehringer Mannheim) was prepared by the manufacturer's instructions. Because the nitrosothiol formed in some reactions (*S*-nitrosodithiothreitol) created very high background absorbance at 340 nm, NADH was also analyzed by strong anion-exchange HPLC (4.6 × 250 mm column; Mac-Mod Analytical, Chadds Ford, PA) with a mobile phase of 25 mM sodium phosphate, pH 7.0, at 1 ml·min^{-1} and detection at 340 nm. NADH eluted at 8 min, and *S*-nitrosodithiothreitol eluted at 4 min.

RESULTS AND DISCUSSION

To characterize the NO-stimulated modification, purified GAPDH was treated with NAD radiolabeled in three different positions (adenine, phosphate, and nicotinamide) with or without SNP, a NO-releasing agent. Labeling of GAPDH was monitored by autoradiography after SDS/PAGE (Fig. 1). GAPDH was modified equivalently with [*nicotinamide*-^{14}C]NAD and [*adenine*-^{14}C]NAD (Fig. 1*A*). Automated analysis (PhosphorImager) of the radioactivity associated with the bands indicated that SNP increased incorporation of radiolabel ≈30-fold and that the amounts of label incorporated into GAPDH from either [*adenine*-^{14}C]NAD or [*nicotinamide*-^{14}C]NAD were within 20%. Equivalent incorporation of both nicotinamide and adenine is not consistent with ADP-ribosylation of GAPDH, in which case only the adenine portion of NAD would be associated with the protein, while nicotinamide would be released.

ADP-ribose is recognized as a reactive species able to combine with protein amino groups and with other, undefined linkages (16–18). To exclude the possibility that the reported 1–5% labeling of GAPDH (9, 11–13) was from a nonenzymatic reaction with ADP-ribose, GAPDH was treated with [*adenylate*-^{32}P]NAD or its breakdown product [*adenylate*-^{32}P]ADP-ribose (Fig. 1*B*). GAPDH was labeled ≈10-fold more with NAD than with ADP-ribose, making it unlikely that the labeling of GAPDH was due solely to a nonenzymatic reaction of ADP-ribose with the enzyme.

Amounts of NAD bound were determined with native GAPDH (total bound NAD) and GAPDH denatured with SDS (covalently bound NAD). After a 60-min incubation with radiolabeled NAD, [*nicotinamide*-^{14}C]NAD, [*adenine*-^{14}C]NAD, and [*adenylate*-^{32}P]NAD were bound to native GAPDH equivalently, at ≈0.65 mol of NAD·mol of GAPDH^{-1} (Table 1). Nitroprusside typically had a slight inhibitory effect on total bound NAD. The amount of NAD covalently bound to GAPDH after denaturation with SDS was much lower, ≈0.006 mol of NAD·mol of GAPDH^{-1} and was increased >3-fold by SNP to ≈0.02 mol of NAD·mol of GAPDH^{-1} (Table 1). The amount of covalently bound NAD was similar with all three radiolabeled species of NAD. Overnight incubations increased the total amount of NAD bound to GAPDH to >2 mol of NAD·mol of GAPDH^{-1}, but the fraction covalently bound was still <5%. Changing the concentrations of SNP (0.001–20 mM) and dithiothreitol (0.001–20 mM) or performing the reaction in the presence of the substrates glyceraldehyde 3-phosphate and inorganic phosphate did not increase the amount of covalently bound NAD above that obtained with 1 mM SNP and 1 mM dithiothreitol.

The extent of covalent NAD binding to GAPDH stimulated by SNP varied in eight different experiments with this method from 10.0 to 22.9 mmol·mol^{-1} (mean ± SD, 16.5 ± 4.8). SNP stimulated the binding of NAD to GAPDH to an apparently greater extent in the SDS/PAGE analysis (30-fold stimulation by SNP) compared with the de-salting column method (3.3-fold stimulation). The difference in apparent

Table 1. Effect of SNP on binding of NAD to GAPDH

	NAD bound, mmol·mol GAPDH^{-1}					
	Before SDS treatment			After SDS treatment		
NAD	Control	+ SNP	% control	Control	+ SNP	% control
[*nicotinamide*-^{14}C]NAD	678 ± 63	530 ± 20	78	7.2 ± 1.4	23 ± 4.2	319
[*adenine*-^{14}C]NAD	613 ± 37	517 ± 12	84	5.2 ± 0.7	18 ± 1.8	346
[*adenylate*-^{32}P]NAD	632 ± 25	572 ± 30	91	6.0 ± 0.2	22 ± 1.1	367

NAD bound to native GAPDH (before SDS treatment) and denatured GAPDH (after SDS treatment) was determined with or without SNP, as described. Data are means of triplicate measurements ± SDs from one experiment representative of three experiments.

stimulation between the methods was due to a much better separation of bound from free NAD by SDS/PAGE versus chromatography over a de-salting column, resulting in a lower background.

Covalent binding of NAD to GAPDH was stimulated by another NO donor, *S*-nitrosodithiothreitol (prepared as described in ref. 19). In an experiment like that of Table 1, covalent NAD binding in the control (4.0 ± 0.3 mmol of NAD·mol of $GAPDH^{-1}$) was stimulated 2.2-fold by 1 mM SNP (significance determined by *t* test; $P < 0.0001$, $n = 6$) and 1.7-fold by 1 mM *S*-nitrosodithiothreitol ($P < 0.0001$, $n = 6$).

Radiolabel incorporated into GAPDH was relatively stable in acid and neutral hydroxylamine; 80% of radiolabel remained, as determined by PhosphorImager analysis. Radiolabel incorporated into GAPDH, however, was sensitive to base (20% remaining) or $HgCl_2$ (10% remaining) (Fig. 2). This pattern of chemical reactivity is similar to that of ADP-ribosylcysteine linkages formed by pertussis toxin and NAD in the α subunits of the guanine nucleotide-binding regulatory proteins G_i, G_o, and transducin (20–25). Although the GAPDH modification was not ADP-ribosylation, the sensitivity to Hg^{2+} may still indicate a linkage with a sulfhydryl group. No NAD–thiol linkage was formed in incubations of SNP and NAD with low-molecular-weight thiols (dithiothreitol, cysteine, or *N*-acetylcysteine), indicating a requirement for the active-site structure of GAPDH.

The product from labeling with either [*nicotinamide*-^{14}C]NAD or [*adenylate*-^{32}P]NAD and released from GAPDH by treatment with $HgCl_2$ eluted in two main peaks on anion-exchange HPLC (Fig. 3). Products with the same retention times were also released by $HgCl_2$ treatment of GAPDH modified with [*adenine*-^{14}C]NAD (data not shown). Although neither product coeluted with standard NAD, this appears effectively to exclude modification of GAPDH by the binding of ADP-ribose and nicotinamide separately to different sites on the enzyme. In the absence of $HgCl_2$, little labeled material was released from GAPDH, indicating the modification of the protein was fairly stable (Fig. 3 *Lower*). The released products were not stable in solution, however, as degradation products were observed in the HPLC eluate. Compounds released from GAPDH by treatment with $HgCl_2$ were incubated with snake venom phosphodiesterase. The products, separated by strong anion-exchange HPLC, appeared to be (*i*) adenosine 5′-monophosphate (retention time = 12 min) and (*ii*) two nicotinamide-labeled compounds (8 min for one product and 9 min for the other) that did not coelute with nicotinamide (6 min), NAD (10 min), nicotinamide ribose phosphate (19–20 min), or NADH (26–28 min). We tentatively conclude that the compounds released from GAPDH by treatment with Hg^{2+} were NAD-like molecules altered in the nicotinamide-ribose-phosphate portion.

GAPDH is thought to be *S*-nitrosylated by NO, probably at the active-site cysteine residue (13). GAPDH activity was inhibited by 1 mM SNP in the presence of dithiothreitol; maximal inhibition was at least 80% at 1 mM dithiothreitol (Fig. 4*B*). Either more or less dithiothreitol resulted in less inhibition of GAPDH activity by SNP. Under conditions where inhibition was 80%, labeling was still <2% (Fig. 4*A*). Labeling, also, was maximal with 1 mM dithiothreitol in the presence of 1 mM SNP, with less labeling at lower and higher concentrations of dithiothreitol. Dithiothreitol, which is required as a reductant for the release of NO from SNP, may also be required as an intermediate carrier of the NO from SNP to GAPDH, in the form of a nitrosothiol (2, 13, 26–28). To determine whether SNP would inhibit GAPDH in the absence of NAD, enzyme exposed to 1 mM SNP/1 mM dithiothreitol for 30 min was passed over a PD-10 desalting column and then assayed without SNP present. Enzyme activity was not significantly inhibited after these treatments, perhaps indicating a limited half-life for the nitrosylated form of GAPDH.

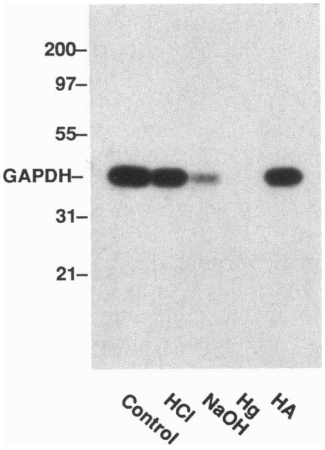

FIG. 2. Release of radioactivity from [^{32}P]NAD/GAPDH labeled in the presence of SNP. GAPDH (160 μg; 22 μM monomer) was incubated with 0.1 mM [^{32}P]NAD (8.5 μCi) in 100 mM sodium Mops, pH 7.0/10 mM EDTA/1 mM dithiothreitol/1 mM SNP (total volume, 0.2 ml) for 1 hr at 30°C. Protein was precipitated with trichloroacetic acid and subjected to electrophoresis. The section of gel between the 28- and 44-kDa prestained standard markers was excised and cut into ≈5 × 5 mm squares. Protein was electroeluted from the gel in an Elutrap apparatus (Schleicher & Schuell) in 20 mM Tris/192 mM glycine/0.1% SDS, pH 8.5, for 4 hr at 200 V. Eluted protein was adjusted to pH 6.0 with 1 M acetic acid. Samples were incubated for 2 hr at 37°C with H_2O (control), 0.2 M HCl (HCl), 0.2 M NaOH (NaOH), 10 mM $HgCl_2$ (Hg; Fluka, Ronkonkoma, NY), or 2 M hydroxylamine hydrochloride (Fisher) in 0.1 M Tris, adjusted to pH 7.0 with NH_4OH (HA). Proteins were precipitated with trichloroacetic acid and subjected to electrophoresis. Radioactivity in the gel was analyzed by autoradiography and by a PhosphorImager. Data are representative of three experiments.

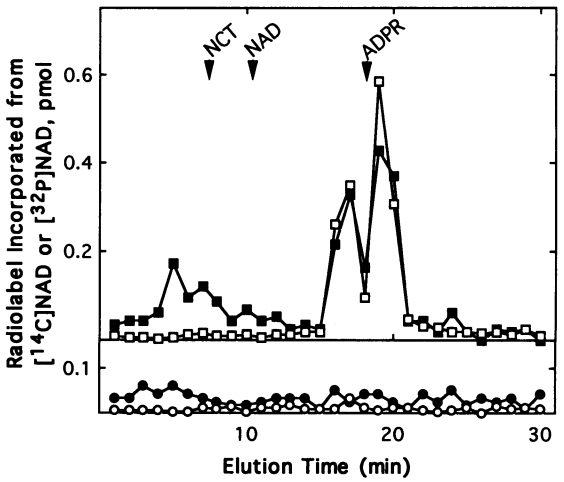

FIG. 3. Ion-exchange separation of the products released by Hg^{2+} from GAPDH labeled with [*nicotinamide*-^{14}C]NAD or [*adenylate*-^{32}P]NAD. GAPDH was incubated with 0.1 mM [*nicotinamide*-^{14}C]NAD (1.05 μCi) or [^{32}P]NAD (8.5 μCi) in the presence of SNP and dithiothreitol, subjected to electrophoresis, electroeluted, and adjusted to pH 6.0 with 1 M acetic acid, as indicated in the legend to Fig. 2. Samples were incubated with 10 mM $HgCl_2$ or with water as a control, for 1 hr at 37°C. Released material was separated from protein by filtration through a Centrifree unit (Amicon), and the filtrate was analyzed by anion-exchange HPLC for radioactivity (14). (*Upper*) Products from $HgCl_2$ treatment of [*nicotinamide*-^{14}C]NAD/GAPDH (■) or [*adenylate*-^{32}P]NAD/GAPDH (□). (*Lower*) Products from control treatment of [*nicotinamide*-^{14}C]NAD/GAPDH (●) or [*adenylate*-^{32}P]NAD/GAPDH (○). Data are from one HPLC run of duplicates in one experiment representative of three experiments. NCT, nicotinamide; ADPR, ADP-ribose.

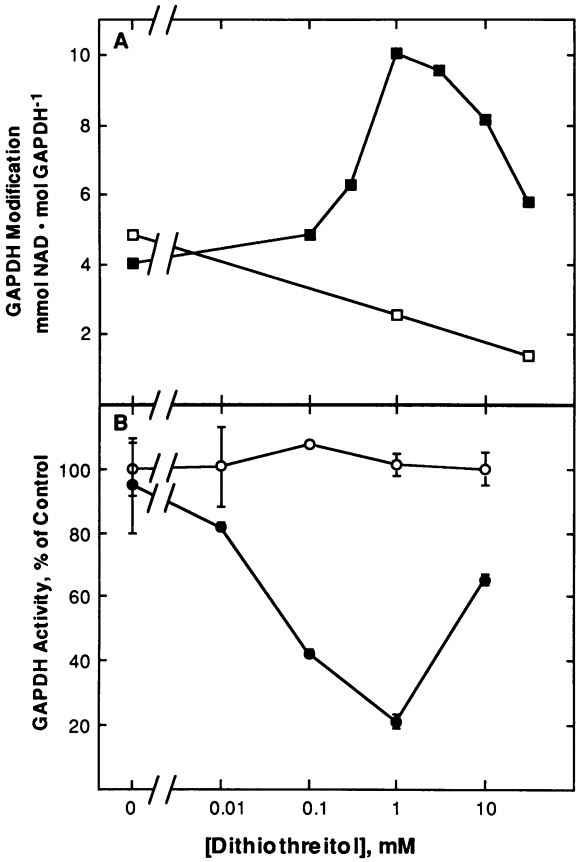

FIG. 4. Effect of dithiothreitol on SNP-stimulated modification and inactivation of GAPDH. (*A*) Modification of GAPDH was done without (□) or with (■) 1 mM SNP with dithiothreitol as indicated for 60 min at 30°C in 0.1 mM [^{32}P]NAD (1 μCi), 0.1 M sodium Mops, pH 7.5/1 mM EDTA (total vol = 0.1 ml). Reactions were stopped by SDS addition to 1% and immersion in boiling water for 5 min. GAPDH was separated from unincorporated NAD, and radioactivity and protein were determined, as described. (*B*) Activity of GAPDH was determined after incubation as in *A* without (○) or with (●) 1 mM SNP and dithiothreitol as indicated. Data are the means of duplicate (*A*) or triplicate (*B*) measurements in one experiment representative of three experiments.

The ability of excess dithiothreitol to protect GAPDH from inhibition and modification may reflect a reversible competition between dithiothreitol and the relevant residue on GAPDH, perhaps the active-site cysteine, for a mobile NO moiety. A precedent exists for a functional effect of NO on the redox state of protein cysteine residues, in the case of the redox modulatory side of the *N*-methyl D-aspartate receptor (19). Apparently, inhibition of GAPDH activity cannot be explained solely by the extent of modification of the enzyme with NAD and is better correlated with the known *S*-nitrosylation reaction of the active-site cysteine residues with NO (13).

Several possibilities may explain the low extent of modification of GAPDH observed in these experiments. The reaction may include an equilibrium between free and GAPDH-attached forms of the NAD-like molecule. However, analysis by HPLC of the unbound fraction revealed the presence of only standard NAD and not of the modified form. Alternatively, the actual chemical step of NAD attachment to GAPDH may be slow, so that modification is stopped as NO is lost from the system through diffusion of NO gas or oxidation to other less reactive inorganic nitrogen oxide species (2).

These results show that modification of a protein by thiol nitrosylation may result in a group with unexpected chemical reactivity—in this case the covalent binding of an NAD species to GAPDH. The NO-stimulated modification of GAPDH with whole NAD reported here may explain a number of the putative "ADP-ribosylation" reactions described in the literature.

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