Posttranslational Modification of Glyceraldehyde-3-phosphate Dehydrogenase by S-Nitrosylation and Subsequent NADH Attachment*

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Nitric oxide (NO)-related activity has been associated with an NAD⁺-dependent modification of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). However, the mechanism by which NO effects covalent attachment of nucleotide and its role in regulation of enzyme activity are controversial. Recent studies have shown that S-nitrosylation of GAPDH (Cys¹⁴⁹) initiates subsequent modification by the pyridinium cofactor. Here we show that NADH rather than NAD⁺ is the preferred substrate. Transnitrosation from active site S-nitrosothiol to the reduced nicotinamide ring system appears to facilitate protein thiolate attack on the enzyme-bound cofactor. This results in attachment of the intact NADH molecule. Moreover, we find that S-nitrosylation of GAPDH is responsible for reversible enzyme inhibition, whereas attachment of NADH accounts for irreversible enzyme inactivation. S-Nitrosylation may serve to protect GAPDH from oxidant inactivation in settings of cytokine overproduction and to regulate glycolysis. NADH attachment is more likely to be a pathophysiological event associated with inhibition of gluconeogenesis.

The versatility of NO as a biological messenger reflects its participation in rich additive and redox chemistry. Pathways of NO oxidation involve reactions with O₂, O₂⁻, and transition metals, which support the formation of surrogates retaining NO-like bioactivity (1, 2). This is exemplified in the case of S-nitrosothiols that are formed in vivo and serve as NO-group donors (3–5). In particular, NO⁺ donation (heterolytic decomposition) appears to be the predominant mechanism of RSNO³ metabolism in many biological systems (6–8). Examples of NO⁺-related activities include allosteric modulation of the N-methyl-D-aspartate receptor involved in neuroprotection (9), the antimicrobial effects of RSNO (10), the inhibition of many sulfhydryl-containing enzymes (11, 12), the activation of p21ras and tissue plasminogen activator, and the down-regulation of transcriptional activators (13–15).

NO-related signal transduction can be broadly classified as cGMP-dependent or mediated through redox signaling events (1). The latter is, perhaps, best exemplified in the regulation of protein function by S-nitrosylation (16). In the case of enzymes that contain critical thiols at their active site, covalent attachment of the NO group leads uniformly to functional attenuation. Examples of enzymes in this category include cathepsin B, aldolase, γ-glutamylcysteiny1 synthetase, aldehyde dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (see Ref. 1 and references therein). Studies on the potential regulation of GAPDH have received particular attention in view of evidence that NO-related activity (nitric oxide synthase activity or NO donors) stimulates an NAD⁺-dependent posttranslational modification of active site thiol in association with the loss of protein function (3, 17, 18). The demonstration of such a modification in cells has led to the proposal that NO induces an ADP-ribosylation reaction reminiscent of that catalyzed by bacterial and mammalian enzymes (19–21). In this reaction, the ADP-ribose moiety of NAD⁺ is transferred to acceptor amino acids with the release of nicotinamide. Studies by Pandol and Fischetti (22) may provide the strongest evidence in favor of a true thioglycosidic linkage induced by NO. More recently, however, this mechanism has been challenged by the demonstration that both the ribose and the nicotinamide moieties of NAD⁺ are incorporated by GAPDH (23). This activity implies linkage of the intact molecule to the active site of the enzyme. Furthermore, inhibition of enzyme activity has seemed to correlate better with the extent of S-nitrosylation than the attachment of [32P]nucleotide, the latter representing only a small fraction of the total protein (23).

We recently probed the mechanism of GAPDH modification using several NO donors. Our studies revealed that NO⁺ transfer to active site thiol is requisite for subsequent modification by [32P]NAD⁺ (3). These data, however, raise a fundamental paradox, as the pathway by which S-nitrosylation facilitates covalent modification by NAD⁺ is not readily apparent. We reasoned, therefore, that NADH rather than NAD⁺ is involved in this reaction, since reduction of nicotinamide would make it susceptible to activation via nitrosative attack. Here we show that 1) S-nitrosylation promotes covalent attachment of reduced nicotinamide; 2) covalent modification by [32P]NADH occurs (largely) via a thionitrocinn linkage; 3) S-nitrosylation of GAPDH accounts for reversible enzyme inhibition, and 4) covalent modification by NADH is responsible for irreversible protein inactivation.
EXPERIMENTAL PROCEDURES

Materials—[^1]^P NAD^+ (800 Ci/mmole) was purchased from DuPont. NEN. SIN-1 was provided by Cassella AG (Frankfurt, Germany). BF_{NO} was purchased from Aldrich, and BF_{NO} was obtained from Fluka. Rabbit muscle GAPDH (80 units/mg) and trypsin (high sequencing grade) were obtained from Boehringer Mannheim. Pertussis toxin A protomer and isocitradehydrogenase (NAD^+ specific, 31 units/mg) were obtained from Calbiochem. Other chemicals including nicotinamide, 1,5'-etheno adenine dinucleotide were of the highest grade of purity made available by Sigma.

Preparation of[^1]^P NAD^+ and nicotinamide-[^1]^C NAD^+—[^1]^P NAD^+ (10 μl, 50 μCi) of[^1]^P NAD^+ (800 Ci/mmole) was incubated with 2 mM MnCl^2, 10 mM isocitrate (pH 7.5), 1 μM cold NAD^+, 22 units of isocitrate dehydrogenase (NAD^+ specific, 31 units/mg), and 100 mM Hepes (pH 7.5) in a total volume of 100 μl at 37 °C for 45 min. Samples were loaded on a nucleosil C18 reverse phase column equilibrated with buffer A (200 mM potassium phosphate buffer, pH 6.0) at a flow rate of 1 ml/min. Nucleotides were separated by elution with buffer B (200 mM potassium phosphate buffer, pH 6.0, 5.0% methanol) using gradient elution: 0–10 min, 70% B; 11–26 min, 100% B; 27–35 min, 70% B at a flow rate of 0.7 ml/min. On-line detection of radioactivity (Ramona) was used to localize peak fractions. Elution of NAD^+ occurred at 15 min and NADH at 20 min. Eluated fractions were collected, and radioactivity was determined using a liquid scintillation counter (PW 4760 from RayTest). 120,000–150,000 cpm/assay were added in GAPDH labeling experiments.

For preparation of[^1]^P NAD^+ and nicotinamide-[^1]^C NAD^+,[^1]^C NAD^+ (41 Ci/mmole) was incubated with 2 mM MnCl^2, 10 mM isocitrate (pH 7.5), 22 units of isocitrate dehydrogenase (NAD^+ specific, 31 units/mg), and 100 mM Hepes (pH 7.5) in a total volume of 100 μl at 37 °C for 45 min. Separation of[^1]^C-labeled NAD^+/NAD^− was performed as described above.

Covalent Modification of GAPDH by[^1]^P NAD^+—[^3]^P NAD^+—Radioactive labeling of GAPDH was performed as previously described (3). Briefly, GAPDH (10 μg/assay) was incubated in 100 mM Hepes buffer (pH 7.5) containing 2.5 mM DTT, 50–200 μM of a NO donor,[^1]^P NAD^+ (120,000 cpm/assay), 10 μM NAD^+, or[^3]^P NAD^+.[^1]^P NAD^+ (120,000 cpm/assay), and 10 μM NAD^−. At indicated time points we usually assayed at 37 °C for 20 min proteins were precipitated with 200 μl of 20% ice-cold trichloroacetic acid, left on ice for 30 min, and then centrifuged for 15 min (13,000 × g) at 4 °C. The resulting pellets were then washed twice with 1 ml of 1% ice-cold water-saturated ether and separated in a 10% sodium dodecyl sulfate-polyacrylamide gel. Radioactivity was quantified using the Phosphorimager system (Molecular Dynamics).

Modification of GAPDH Using Nitrosation—Nitronium-tetrafluoroborate—Stock solutions of BF_{NO} and BF_{NO} were prepared under acidic conditions (pH 2; 0.2 mM HCl) (3). Incubations were carried out with GAPDH (10 μg/assay),[^1]^P NAD^+ (120,000 cpm/assay), 10 μM NAD^+, and 50–100 μM BF_{NO} or BF_{NO}, as described above for the covalent modification of GAPDH. DTT (2.5 mM) was included in some assays.

Modification of GAPDH by nicotinamide-[^3]^C NAD^−—nicotinamide-[^3]^C NAD^− Modification of GAPDH was performed in the presence of 800 μM SIN-1, 2.5 mM DTT, 10 μM[^3]^C NAD^+ (40,000 cpm/assay), or 10 μM nicotinamide-[^3]^C NAD^− (40,000 cpm/assay) as outlined above. For detection of radioactivity, gels were exposed (Phosphorimager exposing cassettes) for up to 30 days.

HgCl2 Cleavage of Pertussis Toxin—[^1]^P NAD^+ treated Proteins and of SIN-1[^1]^P NAD^+ treated GAPDH—Pertussis toxin induced ADP-ribosylation was achieved by incubating human platelet microsomes (80 μg/assay) with 1 μM ATP, 0.1 mM GTP, 10 mM thymidine, 10 μM cold NAD^+ (0.5 μCi/assay), and pertussis toxin A protomer (0.5 μg/assay, activated by treatment with 10 μM DTT for 45 min at 37 °C). Preparation of platelet microsomes was performed according to published procedures (24). Proteins were precipitated with 200 μl of 20% ice-cold trichloroacetic acid and left on ice for 30 min. Following centrifugation (10,000 × g, 15 min) protein pellets were washed twice with ice-cold water-saturated ether. Samples of either pertussis toxin[^1]^P NAD^+ treated proteins or[^1]^P NAD^+ treated GAPDH were resuspended in 100 μl of 100 mM Hepes buffer (pH 7.5) containing 0.5–5 mM HgCl2. Cleavage experiments were carried out for 90 min at 37 °C and followed by protein precipitation (500 μl of 20% trichloroacetic acid). Samples were then processed for detection of protein-bound radioactivity as described above. Controls were treated in the same way, but replaced by NaN_3.

Tryptic Digestion of NAD^+—modified GAPDH—[^3]^P NAD^+ (200 μg) was modified as described above using 20 μM DTT, 5 mM SIN-1, and 200 μM NAD^+ (500,000 cpm/assay). Reaction mixtures (0.5 ml) were incubated for 60 min at 37 °C. Protein was precipitated with 500 μl 50% ice-cold trichloroacetic acid, and incubates were left at 4 °C for 45 min. After centrifugation, pellets were washed twice with ice-cold water-saturated ether. Samples were then resuspended in 1 ml of 50 mM NH_4HCO_3 buffer (pH 7.4) and incubated with 20 μg of trypsin at 37 °C for 4 h. Peptide fragments were separated by reversed phase high pressure liquid chromatography analysis (Nucleosil C18) using gradient elution: 0–10 min, 100% A; 10–60 min, 100% B at a flow rate of 1 ml/min. Buffer A contained H_2O and 0.04% trifluoroacetic acid, while B consisted of 40% H_2O, 60% acetonitrile, and 0.03% trifluoroacetic acid. Fragments were detected by serial UV absorbance and on-line radioactivity measurements. Radioactive fractions were concentrated, and radioactivity was allowed to decline. The fractions were then subjected to commercial peptide sequencing analysis. Based on theoretical predictions, tryptic digestion of rabbit muscle GAPDH should give three fragments containing cysteine residues. The following peptide contains the active site thiol: Cys^546^:IVSNASC^554^TTN^555^LAPLAK.

GAPDH Activity—[^1]^P NAD^+ (1 μg/assay), 1 mM DTT, 10 μM NAD^+, or[^3]^P NAD^+ (200 μg/assay), and up to 200 μM of a NO donor,[^1]^P NAD^+ (120,000 cpm/assay), or[^3]^P NAD^+. Detection of radioactive fractions was performed using a Phosphorimager. Results are representative of three similar experiments.

**FIG. 1.** Modification of GAPDH by[^1]^P NAD^+ and[^3]^P NAD^+.

**FIG. 2.** Time-dependent modification of GAPDH by NADH in the presence of SNP and SIN-1. Modification of GAPDH was carried out as described under "Experimental Procedures" with 10 μM[^1]^P NAD^+ (120,000 cpm/assay). Detection was performed using a Phosphorimager. Data are representative of three similar experiments.
RESULTS

Covalent Modification of GAPDH: NADH versus NAD+. In the presence of [32P]NAD+, SNP stimulated thiol-dependent covalent radiolabeling of GAPDH, confirming previous reports. However, the incorporation of radioactivity was more pronounced when using the pyridinium nucleotide, i.e., [32P]NADH in place of [32P]NAD+ (Fig. 1). Although some nonspecific NO-independent labeling occurred with [32P]NADH, the site of protein attachment was found to be distinct from that supported by S-nitrosylation and could be readily controlled for (see below).

Importantly, NO donors of several different molecular classes were found to induce [32P]NADH-dependent GAPDH labeling. However, the time course of enzyme modification varied among compounds (Fig. 2). For example, SNP-induced labeling was detected at 2.5 min and reached saturation by 5 min, whereas SIN-1 modification occurred at a much slower rate. Radiolabeling was noted after 10 min and required 40 min to achieve levels comparable with those with SNP. Nonspecific labeling was observed with NADH but required reducing conditions, i.e., DTT, and much longer reaction times (i.e., labeling was not detectable during the first 20–30 min).

The pH optimum for nucleotide incorporation was 7.5 for NADH and above 8.5 for NAD+, in agreement with involvement of enzyme (active site) thiolate (Fig. 3). In aggregate, these data are compatible with reports that S-nitrosylation of GAPDH is rate-limiting, since SNP is a better nitrosating agent than SIN-1. We speculate that the higher pH optimum for NAD+ may reflect its more efficient reduction by reduced thiol at alkaline pH (26). Nonspecific labeling by reduced nucleotide is also more prevalent under alkaline conditions.

The amount of radioactivity incorporated into GAPDH (based on equivalent amounts of cold and radioactive labeled nucleotide) was much higher with NADH than with NAD+. Under optimal labeling conditions (10 μM NADH, 2.5 mM DTT, 20 min, n = 16), SIN-1 (200 μM) stimulation led to 1.14 ± 0.37 mol of NADH/mol of GAPDH. Similar rates of incorporation were achieved using 200 μM SNP (1.05 ± 0.15 mol of NADH/mol of GAPDH, mean ± S.D., n = 8; 2.5 mM DTT for 10 min). These results demonstrate the modification of approximately one GAPDH subunit/molecule holoenzyme (GAPDH consists of four identical 39-kDa polypeptide chains), and are to be contrasted with relatively trivial modification by NAD+ (Ref. 23, Figs. 3 and 4).

Posttranslational GAPDH Modification: Mechanistic Considerations—In order to probe the mechanism of nucleotide attachment, GAPDH modification was monitored in the presence of 14C-labeled nucleotide derivatives, specifically [nicotinamide-14C]NAD+ and [nicotinamide-14C]NADH. As shown in Fig. 4, the extent of protein labeling with [nicotinamide-14C]NAD+/NADH was not affected by the presence or absence of NO donors (SNP or SIN-1).

FIG. 3. pH-dependent modification of GAPDH by NAD+ and NADH. Modification of GAPDH in the presence of 10 μM [32P]NAD+ (120,000 cpm/assay) and 10 μM [32P]NADH (120,000 cpm/assay) was determined under the following assay conditions: 100 mM Mes, pH 6.5; 100 mM Tris, pH 7.5; 100 mM Hepes, pH 8.5. SNP and DTT were incubated in each reaction mixture for 20 min. For experimental details see Fig. 1. One of three representative experiments is shown.

FIG. 4. Modification of GAPDH by [nicotinamide-14C]NAD+ and [nicotinamide-14C]NADH. GAPDH labeling was performed for 20 min with SIN-1, DTT, 10 μM [nicotinamide-14C]NAD+ (40,000 cpm/assay) or 10 μM [nicotinamide-14C]NADH (40,000 cpm/assay). Further details are outlined under "Experimental Procedures." Similar results were obtained in two separate assays.

FIG. 5. Pertussis toxin and GAPDH catalyzed reactions using e-NAD+. A, pertussis toxin-catalyzed ADP-ribosylation was carried out as described under "Experimental Procedures" using 10 μM e-NAD+ at 37°C for 40 min. B, GAPDH modification was performed as described above, using 10 μM e-NAD+. Changes in fluorescence were recorded as difference spectra. Experimental details are described under "Experimental Procedures."

FIG. 6. NADH-dependent covalent modification of GAPDH induced by BF4NO and BF4NO2. Modification of GAPDH was performed with 10 μM [32P]NADH (150,000 cpm/assay), DTT, BF4NO, and BF4NO2. Details are given under "Experimental Procedures." This figure is representative of three similar experiments.

FIG. 7. HgCl2 cleavage of ADP-ribose following pertussis toxin treatment and of NADH following NO stimulation. Human platelet microsomes (80 μg/assay) were incubated with 10 μM [32P]NAD+ (0.5 μCi/assay) and pertussis toxin as outlined under "Experimental Procedures." Labeling of GAPDH (10 μg/assay) was performed with 200 μM SIN-1, 2.5 mM DTT, and 10 μM [32P]NADH (200,000 cpm/assay) for 20 min. For HgCl2 cleavage experiments, protein pellets were resuspended in 100 mM Hepes buffer (pH 7.5) and incubated for 90 min with HgCl2. Following protein precipitation, the remaining radioactivity was measured as described under "Experimental Procedures." Data are representative of three experiments.
Posttranslational Modification of GAPDH

Table I

Enzyme inhibition of GAPDH by NADH, NAD⁺, and related compounds

GAPDH (1 μg assay) was incubated with 200 μM SIN-1, 1 mM DTT, and 10 μM of nicotinamide cofactor at 37 °C. GAPDH enzyme activity was measured as described under "Experimental Procedures." Results represent the mean (± S.D.) of three different experiments.

| Cofactor (10 μM)                  | Percentage of inhibition |
|-----------------------------------|--------------------------|
|                                   | 10 min | 20 min | 40 min | 60 min |
| NADH                             | 42 ± 2.8 | 65 ± 2.3 | 75 ± 1.4 | 83 ± 1.2 |
| NAD⁺                             | 20 ± 1.4 | 37 ± 1.4 | 55 ± 0.5 | 68 ± 3.4 |
| β-nicotinamide mononucleotide     | 20 ± 1.0 | 28 ± 0.1 | 45 ± 2.5 | 54 ± 1.5 |
| Nicotinamide                      | 13 ± 2.5 | 21 ± 1.5 | 39 ± 3.1 | 41 ± 1.4 |
| Nicotinamide-N-propylsulfonic acid| 13 ± 1.5 | 15 ± 2.5 | 27 ± 2.1 | 44 ± 2.5 |

Table II

Inhibition of GAPDH by NADH: concentration and time dependency

GAPDH (1 μg assay) was incubated with 200 μM SIN-1, 1 mM DTT and 1 μM, 10 μM, or 100 μM NADH at 37 °C. GAPDH enzyme activity was measured as described under "Experimental Procedures." Results represent the mean (± S.D.) of three different experiments.

| NADH (μM) | Percentage of inhibition |
|-----------|--------------------------|
|           | 10 min | 20 min | 40 min |
| 1         | 26 ± 1.2 | 32 ± 1.4 | 45 ± 2.4 |
| 10        | 42 ± 2.5 | 65 ± 2.5 | 75 ± 1.4 |
| 100       | 70 ± 2.8 | 97 ± 2.4 | 100 ± 1.1 |

14C]NAD⁺ was modest in comparison with that induced by [nicotinamide-14C]NADH. These data emphasize involvement of NADH and suggest that its linkage to protein occurs via the nicotinamide ring.

Experiments performed with ε-NAD⁺ further support the notion that binding involves the intact pyridine cofactor. Upon cleavage of the β-nicotinamide bond in ε-NAD⁺ by pertussis toxin, the fluorescence of the molecule increased, much as described previously for NADase (25). In comparison, modification of GAPDH using ε-NAD⁺ did not result in a change in fluorescence (Fig. 5), even though the substitution for NAD⁺ led to a comparable degree of GAPDH inhibition. For example, modification of GAPDH (10 μg) by 200 μM SIN-1 (2.5 mM DTT) in the presence of either 10 μM NAD⁺ or 10 μM ε-NAD⁺ resulted in a 55 ± 0.5% versus 54 ± 3.5% decrease in enzyme activity relative to controls after 40 min, respectively.

We further reasoned that nitrosation of reduced nicotinamide was required for ring activation. This would then facilitate protein thiolate attack on the nucleotide by increasing its electrophilicity. To test the validity of this mechanism, we first examined the effects of nitrosation-tetrafluoroborate (BF₄NO), a strong NO⁺ donor (Fig. 6). In the presence of DTT, BF₄NO potently induced GAPDH labeling by [32P]NADH. Moreover, the NO⁺-donor nitronium-tetrafluoroborate (BF₄NO₂⁻), which on theoretical grounds should be equally capable of nicotinamide activation (3), resulted in comparable degrees of GAPDH modification (Fig. 6). With both agents, maximal labeling was achieved at concentrations of 50 μM under reducing conditions.

Cleavage experiments with HgCl₂ were then performed in order to confirm that the NADH linkage involved protein thiol groups (i.e. Cys₄⁴⁹ of GAPDH). Specifically, the enzyme preparation was treated with HgCl₂ after covalent modification had been induced with SIN-1. HgCl₂ (5 mM) was found to displace the greater part of the [32P]NADH radiolabel (Fig. 7).

Importantly, radioactivity incorporated by incubating GAPDH with 10 μM NADH and 2.5 mM DTT, i.e. nonspecific labeling, could be readily discriminated from active site modifications due to its resistance toward Hg²⁺ treatment. 5 mM HgCl₂ or 10 mM DTT was unable to remove any such incorporated radioactivity. NO-independent radioactivity, however, was partially cleaved (around 40% decrease) by treatment with 5 mM NH₂OH for 90 min. For comparison, the effects of HgCl₂ were examined on human platelet membranes that had been ADP-ribosylated by treatment with pertussis toxin. As previously shown, HgCl₂ (0.5 mM) removed all the radioactivity. Furthermore, over 90% of the radioactivity remained bound to GAPDH following treatment with hydroxylamine.

To further identify the cysteine residue involved by NO, a tryptic digestion of radiolabeled GAPDH was performed, attachment followed by sequence analysis of the single fragment that contained radioactivity. Amino acid sequencing identified the peptide IVSNAS, after which analysis resisted further cycling. The sequence matches identically with the predicted tryptic digestion fragment containing the active site Cys₄⁴⁹ residue of rabbit muscle GAPDH. Thus, cleavage experiments combined with tryptic digestion strongly suggest that the modification of GAPDH occurs at Cys₄⁴⁹.

Inhibition of Enzyme Activity—In order to explore the relationship among covalent modification of GAPDH, the mechanism of nucleotide attachment, and enzyme activity, we com-
pared the effects of GAPDH inhibition supported by NADH, NAD\(^+\), \(\beta\)-nicotinamide mononucleotide, nicotinamide, and nicotinamide N-propanesulfonic acid (Table I).

In general, GAPDH inhibition appeared to correlate well with the extent of nucleotide incorporation. In particular, enzyme inhibition induced by SIN-1 was greatest with NADH. NAD\(^+\) was significantly less effective and equal in activity to \(\beta\)-nicotinamide mononucleotide. Nicotinamide and nicotinamide N-propanesulfonic acid also exhibited inhibitory effects, although they were the least active compounds. The time- and concentration-dependent effects of NADH are detailed in Table II. Importantly, radiolabel incorporation induced by S-nitrosylation of Cys\(^{149}\) correlated well with loss of enzyme activity, whereas NO-independent modification of GAPDH by NADH did not (data not shown). Inhibition of GAPDH clearly increased with time and with higher concentrations of the nucleotide.

Experiments were then performed to delineate the role of S-nitrosylation via a covalent NADH attachment in the inhibition of GAPDH (Fig. 8). GAPDH was incubated with BF\(_4\)NO for 5 min followed by the addition of either DTT or NADH. GAPDH activity was restored with the addition of DTT (Fig. 8, upper panel) or NADH (Fig. 8, lower panel). Following the addition of BF\(_4\)NO, we observed an initial drop in enzyme activity by 40% compared with untreated controls. Over the next 5 min, enzyme activity partially recovered spontaneously. Thereafter, either 10 mM DTT or 10 \(\mu\)M NADH were added. With the addition of DTT, GAPDH activity was restored to control values (90 ± 2.5%). In contrast, NADH caused a time-dependent irreversible inhibition of the enzyme. By 35 min, only 30% of the initial enzyme activity remained (Fig. 8, upper panel). Using \([\text{\textsuperscript{32}}P]\)NADH, we confirmed that the extent of labeling incorporated by GAPDH paralleled the degree of inhibition following the addition of nitrosating agent (Fig. 8, lower panel). We conclude 1) that S-nitrosylation is responsible for early, reversible enzyme inhibition and 2) that S-nitrosylation promotes subsequent irreversible attachment of NADH to active site thiol.

**DISCUSSION**

Nitric oxide has been associated with a mono-ADP-ribosylation-like reaction in which the pyridinium nucleotide undergoes covalent attachment to the active site thiol of GAPDH. However, the mechanism of this reaction is poorly understood, and its contribution to changes in protein function is controversial. We recently demonstrated that GAPDH modification is stimulated by S-nitrosylation, i.e. NO\(^+\) transfer chemistry rather than reaction of nitric oxide (3). In particular, S-nitrosoylation of the enzyme active site thiol (Cys\(^{149}\)) was found to initiate subsequent covalent modification by NADH\(^+\). In order to rationalize this finding, we reasoned that NAD\(^+\) must first be reduced under in vitro assay conditions (1, 3). Transnitrosation from active site RSNO to the nicotinamide ring could then facilitate protein thiolate attack on the nucleotide (1). Here we show that NO-related activity does indeed depend on the presence of reduced nicotinamide and that NADH rather than NAD\(^+\) is the preferred reaction substrate. Specifically, labeling with NADH is much more efficient than with NAD\(^+\), occurs more rapidly, and correlates better with changes in enzyme activity.

Mechanisms of Covalent Modification of GAPDH: S-Nitrosoylation versus Nucleotide Attachment—Our results suggest that both S-nitrosoylation and covalent attachment of nucleotide are relevant mechanisms of GAPDH modification. The close proximity of the cofactor binding site of GAPDH to active site thiol, evidenced by reports of their participation in a charge transfer complex (27, 28), would be predicted to facilitate nitrosative attack at the C-5 position of the nicotinamide ring (29). Resultant ring activation (increased electrophilicity) would then lead to protein thiolate attack at the C-6 position (Fig. 9, upper pathway).

This proposed mechanism is supported by three findings: 1) that several reduced nicotinamide derivatives can substitute for NADH; 2) that treatment with HgCl\(_2\) liberates the reduced nucleotide from active site thiol; and 3) that binding and inhibition by \(\epsilon\)-NAD\(^+\) occurs without cleavage of the \(\beta\)-glycosidic bond. The involvement of reduced nicotinamide might also explain reports of a NO-associated ADP-ribosylation reaction involving a true thiglycosidic linkage. In this scenario, activation of nucleotide (via transnitrosation), engenders thiolate attack at ribose C-1' by making nicotinamide a better leaving group. This reaction (Fig. 9, lower pathway) may be more viable in other proteins where structural constraints place the RSNO in closer proximity to the sugar moiety than the nicotinamide ring.

GAPDH: Enzyme Inhibition and Covalent Modification—Our studies appear to resolve the controversy over the possible contribution of S-nitrosoylation versus covalent attachment of nucleotide to enzyme inhibition. Both are likely to contribute, albeit under very different conditions. Reversible enzyme inhibition is mediated by S-nitrosylation. This posttranslational
modification may be involved in the regulation of glycolysis seen in cellular systems activated with inflammatory cytokines (30). S-Nitrosylation might also provide a means to protect such thiol-containing enzymes from oxidative inactivation. In contrast, irreversible enzyme inhibition (seen in some cellular systems (31)) is likely to be explained by covalent attachment of NADH. This modification is more likely to be a pathophysiological event associated with inhibition of gluconeogenesis.

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