Thiols Mediate Superoxide-dependent NADH Modification of Glyceraldehyde-3-phosphate Dehydrogenase*

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is covalently modified by NAD in the presence of nitric oxide (NO) and dithiothreitol. Replacement of NAD with NADH in the presence of SIN-1 (3-morpholinosydnonimine) and dithiothreitol increased modification 25-fold. We now demonstrate that in contrast to NO-mediated attachment of NAD, covalent attachment of NADH to GAPDH proceeds in the presence of low molecular weight thiols, independent of NO. Removal of oxygen and transition metal ions inhibited modification, consistent with a role for reactive oxygen species; inhibition by superoxide dismutase, stimulation by xanthine oxidase/hypoxanthine, and the lack of an effect of catalase supported the hypothesis that superoxide, generated from thiol oxidation, was involved. Electrospray mass spectrometry showed covalent linkage of the NAD molecule to GAPDH. Characterization of the product of phosphodiesterase cleavage demonstrated that linkage to GAPDH occurred through the nicotinamide of NADH. Lys-C digestion of GAPDH, followed by peptide isolation by high performance liquid chromatography, matrix-assisted laser desorption ionization time-of-flight analysis, and Edman sequencing, demonstrated that NADH attachment occurred at Cys-149, the active-site thiol. This thiol linkage was stable to HgCl₂. Thus, linkage of GAPDH to NADH, in contrast to NAD, occurs in the presence of thiol, is independent of NO, and is mediated by superoxide.

Superoxide and nitric oxide (NO) are important free radical mediators of host immunity, either through their direct actions or as precursors of other reactive species (1). Nitric oxide is generated from l-arginine by NO synthases (2), and activated phagocytic cells (i.e. macrophages, neutrophils, monocytes, and eosinophils) produce superoxide through single-electron reduction of molecular oxygen (3). The latter process, catalyzed by NADPH oxidase, is pivotal to the production of other superoxide-derived reactive oxygen species (ROS), i.e. hydroxyl radical, singlet oxygen, hydrogen peroxide, and peroxynitrite. Its importance in immunity is best exemplified by chronic granulomatous disease, a condition in which defects in the NADPH oxidase complex result in predisposition to bacterial and fungal infections (4). In addition to enzymatic synthesis, superoxide may be generated from oxidation of reduced flavins, catecholamines, tetrahydrofurofates (3), and low molecular weight thiols, e.g. dithiothreitol (DTT), cysteine, or glutathione (5).

Thiols may either stimulate (6) or inhibit (7, 8) NO-dependent reactions. Stimulatory actions are mostly attributed to their ability to transfer NO via transnitrosation reactions (6, 9), whereas inhibitory actions may result from thiol-mediated superoxide generation (7, 8). Superoxide reacts rapidly with NO to form peroxynitrite (ONOO⁻) (1, 3, 10, 11), which is rapidly degraded at physiological pH (1, 11). In smooth muscle relaxation and neurotransmission, NO, serving as a signaling molecule, reacts with the heme group of guanylate cyclase and activates that enzyme, resulting in an increase in cyclic GMP. DTT is required for guanylate cyclase activation by SNP (6), consistent with the proposal that the vasorelaxing properties of NO are in part mediated through the formation of S-nitrosothiol intermediates (6, 9). These low molecular weight nitrosothiols (e.g. S-nitrocysteine and S-nitrosoglutathione) more closely resemble endothelium-derived relaxing factor than NO itself (12) and may transfer NO to abundant plasma-protein thiols for transport in the form of S-nitrosoalbumin (9, 13) or S-nitrosohemoglobin (14).

NO is lipophilic and diffuses readily through cellular membranes (1, 15), interacting not only with plasma and extracellular proteins, but with cytoplasmic proteins as well. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an abundant glycolytic enzyme with a highly reactive active-site thiol, may represent an intracellular target of NO. It is inactivated by NO through nitrosylation of its active-site cysteine (16, 17) and post-translationally modified by NAD or NADH in the presence of nitrogen oxide species and thiols (17, 18). Replacement of NAD by NADH in the presence of SIN-1 (3-morpholinosydnonimine) and DTT increased the degree of modification from ~1 to 25%. It was therefore concluded that NADH was the preferred substrate and thiols stimulated covalent attachment of NADH via a transnitrosation reaction (18).

The specific aim of this study was to determine whether covalent attachment of NADH to GAPDH, observed in the presence of thiols, was actually mediated by a thiol oxidation reaction, independent of NO. These NO-independent effects of thiols on the modification of GAPDH with NADH were compared with their known NO-dependent effects on GAPDH modification with NAD. The potential role of reactive oxygen and free radical species generated during thiol oxidation was examined using enzymatic and nonenzymatic scavengers of superoxide, H₂O₂, and ROS. The nature of the linkage was examined by chemical and phosphodiesterase treatment. Ultimately, the amino acid acceptor was identified by mass spectrometry and sequencing of HPLC-purified peptides.

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‡ The abbreviations used are: ROS, reactive oxygen species; DTT, dithiothreitol; SNP, sodium nitroprusside; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; DHLA, dihydrolipoic acid; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
EXPERIMENTAL PROCEDURES

Materials

Rabbit muscle GAPDH, alcohol dehydrogenase, lactate dehydrogenase, malate dehydrogenase, superoxide dismutase, and catalase were purchased from Roche Molecular Biochemicals. Snake venom phosphodiesterase was from Worthington. α-NAD, trifluoroacetic acid, hypoxanthine, isocitric acid, isocitrate dehydrogenase, i-cysteine, reduced glutathione, dithiobisethanol, dihydroliolic acid (DHLA), and ascorbic acid were purchased from Sigma. Recombinant ADP-ribosylation factor 6 was a kind gift of Dr. Walter A. Patton (NHBLI, National Institutes of Health, Bethesda, MD).

Methods

Generation and Purification of [32P]NADH—[32P]NADH was generated by reduction of [32P]NAD catalyzed by isocitrate dehydrogenase as described previously (17). HPLC purification was performed using a Waters μBondapak C18 column under isocratic conditions with 50 mM potassium phosphate buffer (pH 7.4) and 5% methanol. Under these conditions, NAD eluted at 10 min and NADH at 15 min.

Modification of GAPDH with NAD and NADH—Rabbit muscle GAPDH, NAD-dependent dehydrogenases, and bovine serum albumin (BSA) (100 µg) were dissolved in 100 µl of potassium phosphate and 5 mM EDTA (pH 7.4) and incubated with 25–50 µM adenylyl-[32P]NAD (1 × 10^6 cpn), [nicotinamide-32P]NADH or [adenine-32P]NADH (100,000 cpm/assay) without or with DTT (0.01–100 mM) as indicated (200-µl total volume). The control modification reactions with NAD were carried out with or without SNP as indicated. After incubation at 37 °C for 60 min or overnight, 20 µl of cold 100% trichloroacetic acid were added, and after further incubation for 30 min on ice, the precipitated protein was pelleted by centrifugation (14,000 × g, 20 min). The protein was suspended in sodium acetate buffer containing 1% SDS, mixed with 2× SDS-PAGE sample buffer, heated in boiling water for 5 min, and subjected to SDS-PAGE on 4–20% gradient gels along with prestained molecular weight markers (Novex). Gels were stained with Coomassie Blue, dried under vacuum, and exposed to x-ray film for 16–24 h.

Purification of Modified GAPDH by HPLC—GAPDH was incubated overnight with NADH in the presence of thiol as described above. The reaction mixture was then dissolved in 6 M guanidine and 0.5 M potassium phosphate (pH 2.5) and loaded on a Vydac C18 reverse-phase HPLC column (218TP54) equilibrated with solution A (HPLC-grade water and 0.05% trifluoroacetic acid). The modified GAPDH was separated from the free reactants by gradient elution with solution B (100 mM acetonitrile and 0.05% trifluoroacetic acid) at a flow rate of 0.20 ml/min; and 20–40% solution B from 25 to 45 min, and then 100% solution B from 45 to 50 min.

Mass Spectrometry—Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Hewlett-Packard Model G2025A instrument using a-cyano-4-hydroxycinnamic acid (Hewlett-Packard Co., Palo Alto, CA) as the matrix. The sample and matrix (0.4 µl each) were mixed on the target and dried under vacuum. The instrument was calibrated with an external standard mixture of peptides (Hewlett-Packard Co.). Electrospray mass spectrometry was performed on a Hewlett-Packard Model G1946A instrument interfaced to a Model 1100 HPLC system using essentially the same chromatographic conditions described above for the purification of modified GAPDH. The flow rate for HPLC was 0.20 ml/min, and the effluent was mixed in a tee with a 1:1 mixture of acetic acid/acetonitrile at 0.1 ml each before introduction into the electrospray chamber (19). Spectra were deconvoluted with Hewlett-Packard Chemstation software (Version 5).

Lys-C Digestion of GAPDH—Modified and unmodified GAPDHs were separated by HPLC as described under “Purification of Modified GAPDH by HPLC.” Samples (50 µg) were mixed on the target and dried under vacuum suspended in 50 µl of 8 M urea and 0.4 M NH4HCO3 (pH 8.6); carboxymethylated; and digested with the protease Lys-C, as described (20), using 4 µg of Lys-C in the final incubation. The reaction mixture was then dissolved in 6 M guanidine and 0.6 M potassium phosphate (pH 2.6) and loaded on a Vydac C18 reverse-phase HPLC column (218TP54) equilibrated with solution A. The peptides were separated by gradient elution with 0–60% solution B over 60 min at a flow rate of 1 ml/min, followed by re-equilibration to 100% solution A. Absorbance at 210 and 258 nm were recorded with a Hewlett-Packard diode-array detector (Model G1315A). HPLC-purified peptides were subjected to MALDI-TOF analysis to obtain their mass and then applied to sample columns for automated sequencing in a Hewlett-Packard Model G1605A sequencer using Version 3.5 sequencing routines.

RESULTS

SNA, a nitrososum (NO+) donor (21), is reported to stimulate modification of GAPDH with NAD (16–18, 29, 30, 36–38). Its effect on the modification of GAPDH with NAD was examined without and with DTT (Fig. 1). As demonstrated previously (17, 18), SNP had little effect on covalent NAD and NADH attachment to GAPDH in the absence of thiol. Addition of 2.5 mM DTT with 0.25 mM SNP enhanced NADH attachment to the same level observed with 2.5 mM DTT alone. Addition of 2.5 mM SNP decreased the incorporation of NADH below that level (Fig. 1), whereas it had further enhanced NAD attachment to GAPDH (data not shown). Similar results were obtained with the NO donors S-nitrosoacetylpenicillamine and S-nitrosothiol, and GAPDH was not significantly modified by NAD in the absence of NO at any pH or DTT concentration (data not shown).

In contrast to NAD, NADH did not require NO for attachment to GAPDH, which was stimulated by increasing concentrations of DTT (Fig. 2, upper panel). A ratio of thiol to NO donor (>10-fold) was uniformly inhibitory for SNP-stimulated NAD attachment (Fig. 2, lower panel) and that stimulated by all other NO donors or NO species examined, i.e., S-nitrosoacetylpenicillamine, S-nitrosothiol, ONOO−, and SIN-1 (data not shown). The dithiols DTT and DHLA stimulated modification with NAD at concentrations ranging from 0.01 to 0.1 mM, whereas >10-fold higher concentrations of monothiols (e.g., L-Cys and glutathione) were required to obtain comparable modification (Fig. 3).

To determine whether thiol oxidation played a role in the modification of GAPDH with NADH, transition metal catalysts and oxygen, which stimulate thiol oxidation (3, 5, 22, 23, 31), were removed (Fig. 4). Removal of trace metal catalysts by 0.5 µM Fe(II) and air restored modification to the level observed in the untreated samples. Glutathione, superoxide and thiyl radical scavenger (24), significantly decreased NADH attachment (Fig. 5), supporting the involvement of free radicals in the modification reaction.

Superoxide dismutase, which catalyzes conversion of superoxide to molecular oxygen and hydrogen peroxide (3), decreased
attachment of NADH to below control levels (Fig. 6). Catalase, in combination with superoxide dismutase, was added to determine whether the inhibitory effect of superoxide dismutase on NADH attachment could be due to superoxide dismutase-mediated H$_2$O$_2$ generation. Catalase, by itself or in combination with superoxide dismutase, did not affect modification with NADH. Hence, H$_2$O$_2$ did not appear to play a role in the linkage of NADH to GAPDH or in the inhibitory effect of superoxide dismutase. Because specific superoxide removal by superoxide dismutase decreased modification with NADH, the effect of enzymatic generation of superoxide was then examined. Xanthine oxidase/hypoxanthine-mediated generation of superoxide replicated the effect of DTT on GAPDH modification with NADH (Fig. 6), consistent with the hypothesis that attachment of NADH to GAPDH may be mediated by superoxide. GAPDH is a member of a family of NAD-dependent dehydrogenases with striking structural similarities in their NAD-binding domains (25). The ability of GAPDH to be covalently modified by NADH in the presence of thiols was compared with that of other NAD-dependent dehydrogenases (Fig. 7, upper panel). DTT-mediated covalent attachment of NADH was seen only with GAPDH, whereas in the presence of NO and thiol, NAD attachment was observed with other dehydrogenases as well as GAPDH.

Reverse-phase HPLC was used to separate the modified from native enzyme by means of a shallow water/acetonitrile gradient (see "Experimental Procedures"). Covalent attachment of NADH to GAPDH resulted in earlier elution (Fig. 8A). Electro spray mass spectrometry analysis demonstrated an increase in mass of the early eluting peak by 661 mass units, in good agreement with the mass of NADH (Fig. 8B). To determine the site of NADH attachment to the enzyme, GAPDH modified with [14C]NADH labeled in the nicotinamide or adenine moieties was incubated with snake venom phosphodiesterase as described under "Methods." Radiolabel was removed from the enzyme modified with [adenine-14C]NADH and not from the enzyme modified with [nicotinamide-14C]NADH, consistent with linkage through the nicotinamide moiety of NADH (data not shown).

**FIG. 2.** Effect of DTT on the modification of GAPDH by NAD and NADH. GAPDH was incubated at 37 °C for 60 min at pH 7.4 with the indicated concentrations of DTT and 50 μM [32P]NADH in the absence of a NO donor (upper panel) or 50 μM [32P]NAD plus 1 mM SNP (lower panel). Data are representative of >12 experiments.

**FIG. 3.** Effect of low molecular weight thiols on the modification of GAPDH by NADH. GAPDH was incubated at 37 °C for 60 min with 50 μM [32P]NADH and the indicated concentrations of DTT, l-Cys, GSH, or DHHLA. Data are representative of four experiments.

**FIG. 4.** Effects of metal chelator treatment, re-addition of ferrous chloride, and anaerobic conditions on the modification of GAPDH with NADH. GAPDH was incubated for 30 min at 37 °C with or without 25 mM DTT or 50 mM l-Cys, with or without 10 μM FeCl$_2$ as indicated, and 50 μM [32P]NADH (200-μl total volume) in buffer that had been treated overnight with a metal chelator resin (Chelex 100, Bio-Rad) or in the usual buffer (not treated with Chelex 100), as indicated. Chelex 100-treated HPLC water was also used to prepare DTT, l-Cys, and FeCl$_2$ solutions, and all HPLC equipment was washed overnight with Chelex 100-treated solutions. Trichloroacetic acid (20 μl, 100%) was added after 30 min. To determine the effect of oxygen or its lack on NADH attachment, GAPDH was incubated with DTT or l-Cys and [32P]NADH (as described above) in buffer not treated with Chelex 100 under aerobic or anaerobic conditions (obtained by bubbling buffer with nitrogen gas for 30 min before and continuously during incubation). After 30 min, 100-μl samples were removed and mixed with 10 μl of 100% trichloroacetic acid. Atmospheric oxygen concentration was restored in the remaining 100-μl reactions by bubbling air for 30 min (last lane) before proteins were precipitated with trichloroacetic acid, separated by SDS-PAGE, and detected by autoradiography. Data are representative of three separate experiments with duplicate assays.
Superoxide-mediated Modification of GAPDH

In contrast, the NADH linkage to GAPDH (Fig. 9, middle panel) was stable. It differed also from the ADP-riboylarginine linkage formed by cholera toxin (Fig. 9, lower panel).

Lys-C digestion of modified GAPDH allowed the isolation of a new peptide, not found among peptides from native GAPDH, with higher relative absorbance at 258 nm (Fig. 10). The molecular weight by MALDI-TOF of the peptide from native GAPDH was that of the expected Lys-C peptide IVSNASCTT-NCLAPLAK; whereas the modified GAPDH peptide had an additional 103 mass units, corresponding to the molecular weight of nicotinamide. Analysis of NAD and NADH under the same MALDI-TOF conditions demonstrated that nucleotide cleavage occurred between the nicotinamide and ribose moieties; hence, the increased mass of the modified peptide was consistent with nucleotide attachment, followed by release of the ADP-ribose portion, under MALDI-TOF conditions, leaving nicotinamide attached to the peptide.

The peptide comprising residues 143–159 (IVSNASCTT-NCLAPLAK) was identified in both the modified and native peptide fractions by automated Edman sequencing. Cycles 7 and 11 in the control peptides yielded carboxyamidomethylcysteine, resulting from alkylation by iodoacetamide. The modified peptide released carboxyamidomethyllysteine in cycle 11 and nothing in cycle 7, consistent with covalent attachment of the nucleotide cofactor to active-site Cys-149.

DISCUSSION

GAPDH is an abundant glycolytic enzyme with a highly reactive active-site thiol and hence is a potential in vivo biological target of ROS. The enzyme, which is inactivated by superoxide (27) and NO (16, 17, 28), can be covalently modified by NAD in the presence of NO (17, 18). As reported here, post-translational modification of GAPDH with NADH, as opposed to that with NAD, is stimulated by thiols, possibly through superoxide, and is independent of NO. Thiols, in the presence of NO, facilitate modification with NAD, probably via transfer of the nitroso group from the NO donor to the active-site thiol in a transnitrosation reaction (18, 29). This reactive active-site intermediate is believed to attack the nicotinamide moiety of NAD, leading to covalent NAD attachment (18). An equimolar ratio of thiol to NO donor was important, and when exceeded, modification was decreased (Fig. 2), consistent with NO transfer to low molecular weight thiols and competition with cysteine 149 of the enzyme (17, 30). Modification of the enzyme with NAD was not observed in the absence of a NO donor. Modification with NADH, as opposed to NAD, proceeded in the absence of NO (Figs. 1–3); it was greater at higher thiol concentrations (Fig. 2) and preferentially stimulated by thiols (Fig. 3).

Thiols undergo oxidation with resultant concentration- and time-dependent generation of superoxide (3, 5, 7, 22, 23, 31). Dithiols (e.g. DTT and DHLA) undergo oxidation more readily than do monothiols (e.g. l-Cys and GSH) (31), consistent with the greater extent of modification observed with these compounds. Among dithiols, a smaller distance between thiol groups increases the rate of oxidation. DHLA is hence more efficient than DTT; among the monothiols, cysteine oxidizes more rapidly than does GSH (31). The extent of GAPDH modification with NADH stimulated by these compounds was parallel with their reported rates of oxidation (23, 31). A key step in thiol oxidation is electron transfer from the thiolate to a transition metal ion in its higher oxidation state, which then reacts with molecular oxygen, generating superoxide and maintaining the catalytic cycle (3, 5, 22, 23, 31).

In agreement with the role of thiol oxidation in covalent NADH attachment, modification was decreased after removal of transition metal...
and oxygen and restored after re-addition of FeCl$_2$ and oxygen (Fig. 4).

Oxidation of thiols to disulfides in aerobic solution results in reduction of molecular oxygen to superoxide. Other superoxide-derived ROS (i.e. H$_2$O$_2$ and hydroxyl radical (HO)) may be generated (3, 23), which could potentially mediate covalent NADH attachment. To identify the relevant ROS, specific scavengers were used. Catalase did not affect NADH attachment, excluding H$_2$O$_2$ as a mediator, whereas inhibition of modification by superoxide dismutase was consistent with a role for

FIG. 8. Effect of covalent NADH attachment on the HPLC elution (A) and spectrum (B) of GAPDH. GAPDH was incubated overnight with 50 μM NADH; the modified and native enzymes were separated by reverse-phase HPLC in a shallow H$_2$O/acetonitrile gradient as described under “Purification of Modified GAPDH by HPLC,” with absorbance recorded at 210 and 258 nm with a diode-array detector (Hewlett-Packard Model G1315A), followed by electrospray mass spectrometry as described under “Methods” (A). The UV absorbance spectra of the early eluting (●●●) and late eluting (○○○) peaks and the difference spectrum (——) are compared (B). mAU, milli-absorbance units.

FIG. 9. Chemical stability of NAD and NADH linkages to GAPDH compared with that of the ADP-riboseyl linkage formed by cholera toxin. Cholera toxin (CT) and activated ADP-ribosylation factor 6 (ARF) were auto-ADP-ribosylated as described previously (34). GAPDH was modified with NAD or NADH as described under “Methods,” followed by incubation for 2 h at 37 °C with H$_2$O (control (C)), 0.2 M HCl, 0.2 M NaOH (OH), 10 mM HgCl$_2$ (Hg), 2 M neutral hydroxylamine hydrochloride (HA) in 0.1 M Tris (adjusted to pH 7 with NH$_4$OH), or 0.15 M NaCl. Protein was precipitated with 10% trichloroacetic acid and subjected to SDS-PAGE, followed by autoradiography. Data are representative of three experiments with duplicate assays.
superoxide (Fig. 6). This possibility was confirmed by reproduction of the stimulatory effects of thiols with enzymatic (xanthine oxidase/hypoxanthine) superoxide generation in the absence of NO (Fig. 6).

The chemical nature of the nucleotide-peptide linkage between NADH and GAPDH was investigated by phosphodiesterase treatment and characterization of its chemical stability. Incubation of NADH with snake venom phosphodiesterase results in cleavage of the pyrophosphate bond, with formation of nicotinamide nucleotide and AMP. Phosphodiesterase removed the radioactive label from GAPDH modified with [adenine-14C]NADH, and not from the [nicotinamide-14C]NADH-modified enzyme, consistent with attachment of NADH through the nicotinamide moiety. MALDI-TOF demonstrated that the modified peptide was 103 Da larger in molecular size than the original, corresponding to the nicotinamide moiety of NADH.

Chemical stability of a nucleotide-peptide linkage has been used extensively to aid in the identification of amino acid acceptors for ADP-ribose, e.g. in the linkages found in reactions catalyzed by bacterial toxins (32) and mammalian ADP-ribosyltransferases (33). Cholera toxin catalyzes the ADP-ribosylation of arginine residues and its own automodification with ADP-ribose (32, 34). This nucleotide-peptide linkage is sensitive to hydroxylamine (34), whereas the ADP-ribosylcysteine linkage, formed in guanine nucleotide-binding proteins by pertussis toxin, is labile to HgCl2 (Fig. 9) (35). Extrapolation from the ADP-ribosylation data led to the proposal that cysteine 149 of GAPDH is the amino acid acceptor for NAD/H, based on the HgCl2 sensitivity of the linkage (18) and on the reduction in modification observed after treatment of the enzyme with N-ethylmaleimide (36) or konic acid (37), which blocks free sulphydryl groups (38). In a prior attempt at amino acid sequencing, the tryptic peptide IVSNAS was identified as containing the radioactive nucleotide, but reportedly, further cycling was not possible (18). In our analysis, the chemical stability of the NADH-GAPDH linkage (Fig. 9) suggested a bond different from those introduced by NO or bacterial toxins, with uniform resistance to all conditions tested. Identification of cysteine 149 as the amino acid acceptor was therefore an unexpected finding. Our data suggest that in the presence of superoxide, NADH establishes a linkage to cysteine 149 of GAPDH that appears to involve the nicotinamide moiety and is chemically distinct from the ADP-ribosyl linkages established by ADP-ribosyltransferases or bacterial toxins and the NAD linkage stimulated by NO.

Thiols generate superoxide via oxidation (3, 5, 7, 22, 23, 31), a role for thiols not previously recognized in these post-translational modification reactions. It had been assumed that modification of GAPDH with NAD and NADH proceeded through analogous mechanisms, with NO acting as mediator and NADH as the preferred substrate (18). We demonstrate, however, that in contrast to modification with NAD, modification with NADH is independent of NO and results from concentration- and time-dependent oxidation of low molecular weight thiols with resultant reduction of molecular oxygen to superoxide. The reactive cysteine 149 thiol moiety hence may be involved in multiple types of reactions, including nitrosylation by NO, NO-stimulated modification with NAD via a HgCl2-sensitive bond, and superoxide-mediated modification with NADH to establish the nicotinamide moiety in a HgCl2- and hydroxylamine-stable linkage.

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FIG. 10. Reverse-phase HPLC elution of Lys-C peptides from intact GAPDH by absorbance at 210 nm (A) and at 258 nm (C) and modified GAPDH monitored at 210 nm (B) and at 258 nm (D). Shown are the results with native GAPDH (control) and GAPDH (200 μg) modified overnight with 50 μM NADH and 5 mM DHLA (150-μl total volume). 50-μg samples were carboxamidomethylated and digested with Lys-C protease, followed by reverse-phase HPLC separation as described under “Methods.” The open arrow represents the parent peptide; the closed arrow indicates the modified peptide. Data are representative of those obtained in four separate experiments. mAU, milliabsorbance units.
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