Nitric Oxide-independent, Thiol-associated ADP-ribosylation Inactivates Aldehyde Dehydrogenase*

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Nitric oxide inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase and stimulates NAD-dependent autodigestion of a cysteine (Dimmel, S., Lottepeich, F., and Brune, B. (1992) J. Biol. Chem. 267, 16771-16774). Another NAD-utilizing dehydrogenase that has a catalytic cysteine, aldehyde dehydrogenase (ALDH), was also inhibited by nitric oxide. Unlike glyceraldehyde-3-phosphate dehydrogenase, ALDH was modified in a nitric oxide-independent process by ADP-ribose, but not by NAD. Modification, which proceeded to >2 mol ADP-ribose/mol ALDH, was associated with an exponential decrease in enzyme activity to less than 10% of control. Two types of evidence suggested modification of the ALDH-active site: 1) ADP-ribose inhibited ALDH competitively (Kₐ = 0.46 mM) with respect to NAD (Kₐ = 0.11 mM) in brief incubations and 2) the presence of substrates protected ALDH from both modification and inhibition by ADP-ribose. The ALDH-ADP-ribose bond was sensitive to base and mercuric ion and stable to acid and neutral hydroxylamine, properties shared with the ADP-ribosylcysteine linkage synthesized enzymatically by pertussis toxin. These data demonstrate a novel means of inactivation of an NAD-dependent enzyme, namely the affinity-based modification of the enzyme NAD-site by ADP-ribose, and suggest that nonenzymatic ADP-ribosylation may be responsible for modification of cysteine residues.

Enzyme-catalyzed ADP-ribosylation is a post-translational regulatory mechanism that occurs at a number of amino acid side chains, including carboxyl groups, arginine, cysteine, asparagine, and diphthamide, dictated by the specificity of the ADP-ribosyltransferase (1-3). Nonenzymatic ADP-ribosylation also occurs at several different residues, primarily at lysine amines through Schiff base linkage (4), but also at other amino acids (5). Previously described examples of the nonenzymatic reaction of ADP-ribose with protein appear to be nonspecific, as the target proteins are among the most abundant in the cell, and with a low stoichiometry of ADP-ribose incorporation (4, 6). In the cell, ADP-ribose is the product of the hydrolysis of NAD (7), mono- and poly-ADP-ribosylated proteins (8, 9), and cyclic ADP-ribose (10) and is known to be present in erythrocyte cytosol at 0.45 μM (11). ADP-ribose is degraded by both specific (12, 13) and nonspecific (14) pyrophosphatases in animal cells.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) has been implicated recently as a target for regulation by nitric oxide, a novel biological messenger (15-21). Nitric oxide stimulates an NAD-dependent modification of glyceraldehyde-3-phosphate dehydrogenase at a low stoichiometry (≈5%) possibly at the active-site cysteine (15, 17, 18). Conversely, NO inhibits glyceraldehyde-3-phosphate dehydrogenase activity to a much greater extent (≈80%), probably due to S-nitrosylation of the active-site thiol (16, 18, 21, 22). The nitric oxide-stimulated modification, referred to as auto-ADP-ribosylation (15-19), was more recently shown to be a novel linkage of NAD to glyceraldehyde-3-phosphate dehydrogenase (22).

The experiments reported here demonstrate a different mechanism of NAD-associated modification of a dehydrogenase cysteine residue. Aldehyde dehydrogenase (ALDH; EC 1.2.1.5), another NAD-dependent dehydrogenase with an active-site cysteine (23-25), was inactivated by stoichiometric, thiol-associated modification by ADP-ribose, independent of the presence of nitric oxide.

EXPERIMENTAL PROCEDURES

Materials—ADP-ribose and β-NAD were purchased from Sigma; [adenylate-32P]NAD (30 Ci mmol⁻¹) from Du Pont-New England Nuclear; ALDH (Saccharomyces cerevisiae mitochondrial isozyme) and Triton X-100 from Boehringer Mannheim; acetaldehyde and sodium nitroprusside from Fisher; dithiothreitol from ICN (Cleveland); hydroxylamine hydrochloride and mercuric chloride from Fluka Chemicals (Ronkonkoma, NY); and PD-10 columns from Pharmacia LKB Biotechnology Inc.

Preparation of Radiolabeled ADP-ribose—[adenylate-32P]ADP-ribose was prepared by hydrolysis of [adenylate-32P]NAD with an NADase purified from the particulate fraction of rat brain to >30 μmol (min·mg⁻¹) as described elsewhere (26).

ADP-ribosylation of ALDH—ALDH was dissolved at 5 mg·ml⁻¹ in 100 mM MOPS, pH 7, 100 mM KCl, 5 mM EDTA, 10 mM DTT, 1 mM PMSF, 29% (v/v) glycerol (ALDH buffer). PMSF was present to inhibit a protease activity present in some lots of ALDH. ALDH was ADP-ribosylated in reactions containing 6.5 μM ALDH tetramer (1.5 mg·ml⁻¹) and 1 mM [32P]ADP-ribose (approximately 0.1 μCi·mmol⁻¹) in ALDH buffer (total volume = 0.1 ml). Following incubation at 30 °C for the indicated times, samples (0.01 ml) were transferred to tubes containing 0.24 ml of PD-10 buffer (100 mM sodium acetate, pH 6, 1 mM EDTA, 1 mM PMSF, 0.1% Triton X-100) and applied to PD-10 columns equilibrated with 35 ml of PD-10 buffer. Columns were washed consecutively with 0.25-, 0.5-, and 1.75-ml portions of 2-(N-morpholino)ethanesulfonate; 3-[N-tris(hydroxymethyl)methylamino]propanesulfonate; MOPS, 3-(N-morpholino) propane sulfonate; FMSF, phenylmethylsulfonyl fluoride; TAPS, 3-[N-tris(hydroxymethyl)methylamino] propane sulfonate.

* The abbreviations used are: ALDH, aldehyde dehydrogenase; ADP-ribose, adenosine-diphosphoribose; SNP, sodium nitroprusside; DTT, dithiothreitol; MES, 2-(N-morpholino)ethanesulfonate; MOPS, 3-(N-morpholino)propanesulfonate; FMSF, phenylmethylsulfonyl fluoride; TAPS, 3-[N-tris(hydroxymethyl)methylamino]propane sulfonate.

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RESULTS AND DISCUSSION

Incubation of a brain homogenate with \(^{32}\)P]NAD or \(^{32}\)P]ADP-ribose resulted in labeling of several proteins, including some that were sensitive to HgCl\(_2\), suggesting a thiol-associated linkage (28-30). A ~53-kDa brain protein that was labeled with ADP-ribose in a Hg\(^{2+}\)-sensitive linkage was purified by successive column chromatography steps, subjected to micro-sequencing, and identified as the mitochondrial isozone of ALDH. The sequence obtained was of a tryptic peptide, which perfectly matched amino acids 476-489 of the deduced amino acid sequence from the bovine isozyme cDNA (31). The sequence was: Glu-Leu-Gly-Glu-Tyr-Gly-Glu-Ala-Asp-Thr-Glu-Val-Lys. Previous studies revealed that several mitochondrial proteins react with free ADP-ribose, including a 36-kDa inner membrane protein, and a 52-kDa matrix protein (5, 32), raising the possibility that the ADP-ribosylated matrix protein described previously is ALDH.

The activity and NAD-dependent modification of a different dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, were affected by NO (15-19, 22), so the effects of NO on the activity and modification of ALDH were assessed. ALDH activity was stimulated by DTT, with maximal activity at 1-10 mM DTT (Fig. 1A). DTT is required for SNP to release its nitric oxide (21). In the presence of 10 mM DTT, SNP inhibited ALDH activity (Fig. 1B). The effects of SNP and another nitric oxide-donor, S-nitroso-dithiothreitol (prepared as in Ref. 33) on the activity of ALDH and glyceraldehyde-3-phosphate dehydrogenase are shown in Table I. The activity of both enzymes was inhibited by the two nitric oxide donors, and in all cases excess DTT reduced the extent of inhibition. These data are consistent with a reversible nitrosylation of the active site thiol, which inactivates the enzyme (18, 21).

ALDH was modified to a much greater extent with [adenylate-\(^{32}\)P]ADP-ribose than with [adenylate-\(^{32}\)P]NAD (modification with NAD was 4 ± 1% of that obtained with ADP-ribose) (Fig. 2). The NO donor SNP had little effect on modification of ALDH; modification by ADP-ribose was decreased slightly by SNP to 80 ± 5% of control (n = 8), and modification with NAD was unaffected (115 ± 37% of control, n = 8). These results are in complete contrast to the modification of glyceraldehyde-3-phosphate dehydrogenase, in which case NO stimulated modification by NAD (15-19, 22), and ADP-ribose modified the enzyme to a much lesser extent (19, 22).

The interaction of ADP-ribose with ALDH was characterized via NAD kinetics with ADP-ribose as inhibitor. ADP-ribose from 0.2 to 1 mM inhibited ALDH activity competitively with respect to NAD in 20 min reactions, with no apparent V\(_{\text{max}}\) effect detected in the Eadie-Hofstee plots (Fig. 3A). A secondary plot of the Eadie-Hofstee data (slopes versus ADP-ribose concentration) was linear for ADP-ribose from 0 to 1 mM and yielded a K\(_v\) value of 0.11 mM for NAD and a K\(_v\) value of 0.46 mM for ADP-ribose (Fig. 3B). Either higher ADP-ribose concentrations (as in the experiment with 2 mM ADP-ribose shown in Fig. 3A) or longer incubations (data not shown) resulted in mixed-type inhibition with a pronounced formalism of \(K_v = \frac{2}{3}\) from one experiment representative of three.

| Table I |

Effect of nitric oxide-donating compounds on the activity of ALDH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) without or with excess DTT

| Reaction additions | GAPDH activity | ALDH activity |
|--------------------|----------------|----------------|
| No addition        |                |                |
| 10 mM DTT          |                |                |
| 1 mM DTT           | 100 ± 1.5      | 100 ± 1.9      |
| 1 mM SNP/1 mM DTT  | 83 ± 1.9       | 83 ± 1.9       |
| % of control       |                |                |
| No addition        | 100 ± 1.5      | 100 ± 1.9      |
| 1 mM S-nitroso-DTT | 86 ± 2.0       | 86 ± 2.0       |
| 1 mM SNP/1 mM DTT  | 83 ± 1.9       | 83 ± 1.9       |

ADP-ribose was assayed as described under "Experimental Procedures," with (A) DTT, or with (B) SNP plus 10 mM DTT. Data are means ± S.D. of triplicate measurements from one experiment representative of three.
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**ADP-ribosylation of Aldehyde Dehydrogenase**

![Image](image-url)

**SNP:** $- + - +$

**$[^{32}P]:$ NAD ADPR**

**Fig. 2.** Modification of ALDH incubated with $[^{32}P]$NAD or $[^{32}P]$ADP-ribose with or without nitric oxide. ALDH was incubated with 0.1 mM $[^{32}P]$NAD or $[^{32}P]$ADP-ribose, as described under “Experimental Procedures,” with or without 1 mM SNP. Protein was precipitated with 10% trichloroacetic acid, suspended in SDS-PAGE sample buffer, and subjected to electrophoresis in a 12% acrylamide gel followed by autoradiography or automated quantitative analysis (PhosphorImager, Molecular Dynamics, Sunnyvale, CA). Data are representative of eight experiments.

$\pm 0.7 \text{ h (mean \pm S.D., } n = 6).$ Correlation of ALDH activity with the extent of ADP-ribosylation is shown in Fig. 5. The $\sim 90\%$ inhibition observed at $\sim 2 \text{ mol ADP-ribose/mol ALDH}^{-1}$ is consistent with the property of “half-of-the-sites reactivity” in the ALDH homotetramer.

Consistent with ADP-ribosylation occurring at the active site of ALDH, both ADP-ribose-mediated modification (Fig. 6A) and inhibition (Fig. 6B) of the enzyme were decreased in the presence of substrates. NAD (10 mM) decreased ADP-ribosylation and also slowed inhibition of ALDH to a half-time of 7.6 h, compared to a half-time of 2.7 h with ADP-ribose alone. Acetaldehyde (20 mM) was less effective at protecting ALDH but consistently decreased ADP-ribosylation and slowed the inhibition (half-time = 3.2 h).

The modification of ALDH ADP-ribosylated to 1.8 mol ADP-ribose/mol ALDH$^{-1}$ was stable to acid and neutral hydroxylamine but sensitive to base and mercuric ion (Table II). This pattern of sensitivities, especially the sensitivity to the mild treatment with HgCl$_2$, is strongly indicative of an ADP-ribosylcysteine linkage (28–30).

ADP-ribosylation and inhibition of ALDH were markedly pH-dependent. Modification increased with increasing pH from pH 5.5 to 7.5 and then was constant up to pH 9. ADP-ribosylated ALDH was $>90\%$ sensitive to HgCl$_2$ over the entire pH range, indicating the presence of mainly ADP-ribosylcysteine linkages. Incubation with 1 mM ADP-ribose for 2 h inactivated ALDH by 55–60% at pH 7 and above (Fig. 7). Inactivation increased from pH 5 to 7, with an apparent $pK_a$ value of $\sim 6$.

Several dehydrogenases and other enzymes, both with and without active-site cysteine residues, were incubated with ADP-ribose and the extent of modification measured (Table III). ALDH was the most reactive acceptor protein of the group, and with a linkage $>90\%$ sensitive to HgCl$_2$. Other

**Fig. 3.** Effect of ADP-ribose on ALDH activity. A, ALDH activity was assayed as described under “Experimental Procedures,” except that incubation time was 20 min, and the NAD concentration was varied from 0.03 to 0.5 mM in reactions with no addition (○), with 0.2 mM (○), 0.5 mM (□), 1 mM (●), or 2 mM (△) ADP-ribose. B, a secondary plot of data from (A) with slopes from the Eadie-Hofstee plot plotted versus ADP-ribose concentration from 0 to 1 mM. The $K_i$ value was obtained from the slope of this line. Lines are linear best-fit lines from the plotting program. Data are means of duplicates in one experiment representative of three.

**Fig. 4.** Modification of ALDH and inhibition of activity by $[^{32}P]$ADP-ribose. ALDH was incubated with [adenylate-$^{32}P$]ADP-ribose as described under “Experimental Procedures” for the indicated time before assay of protein-bound radioactivity (○) and ALDH activity (●). ALDH incubated without ADP-ribose retained full activity for 50 h. Data are means of duplicates in one experiment representative of six.
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**TABLE II**

**Stability of the ALDH-ADP-ribose linkage**

| Treatment | ALDH-ADP-ribose | pmol | %
|-----------|----------------|------|---
| Control   | 4.8            | 100  |
| HCl       | 4.9            | 101  |
| NaOH      | 0.1            | 1.3  |
| HgCl₂     | 0.3            | 6.2  |
| NH₂OH     | 5.1            | 106  |

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**FIG. 5.** Correlation of ALDH activity with the extent of ADP-ribosylation. Data from an experiment like that shown in Fig. 4 are plotted as ALDH activity versus ADP-ribose incorporated.

**FIG. 6.** Modification and inhibition of ALDH incubated with ADP-ribose with and without substrates. ALDH was incubated with [³²P]ADP-ribose with no addition (○), with 10 mM NAD (□), or with 20 mM acetaldehyde (●) for the indicated time before assay of ADP-ribose incorporated (A) or ALDH activity (B). Data are means of duplicates in one experiment representative of three.

**FIG. 7.** Effect of pH on the ADP-ribose-induced inhibition of ALDH activity. ALDH (0.1 mg ml⁻¹) was incubated for 2 h with 1 mM ADP-ribose in 100 mM buffer (total volume = 0.05 ml). The buffers used were the potassium forms of citrate (○), MES (□), MOPS (●), and TAPS (△). For assay of ALDH activity samples were diluted with ALDH buffer so that the concentration of ADP-ribose was 0.5 μM. ALDH lost activity during incubations at pH < 6, but was uniformly stable in incubations from pH 6 to 9. Data are the means of duplicates in one experiment representative of three.

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The present work demonstrates a specific active-site thiol-associated modification of ALDH by a novel affinity reagent, ADP-ribose. The modification of an essential cysteine in ALDH appears to be a special case of an enzyme with a reactive cysteine in a coenzyme site that has an affinity for ADP-ribose. Whether ALDH reacts with ADP-ribose in the mitochondria is unknown. Considering that the reaction of 1 mM ADP-ribose with ALDH was rather slow, on the order of hours, and not knowing the concentration of ADP-ribose in the mitochondrial matrix, it is difficult to guess to what extent this enzyme may be ADP-ribosylated in vivo. If the modification were stable chemically, and not removed by the action of an ADP-ribosylcysteine hydrolase, then the modification of ALDH could accumulate over time, dependent on turnover of the enzyme.

ADP-ribose has been shown to react with free cysteine to form a thiazolidine linkage in which both the amino and sulfhydryl groups of cysteine are linked to ADP-ribose (26). Presumably, the ADP-ribose linkage with ALDH is not of the thiazolidine type, because the amino group of the ALDH cysteine would be included in an amide linkage, and therefore not available for thiazolidine formation. Rather, the ADP-ribosylcysteine linkage in ALDH resembles that synthesized by pertussis toxin from NAD in the guanine nucleotide-binding regulatory proteins (28, 29). The chemical reactivity of the ADP-ribose bond in ALDH is also consistent with a thioacetal linkage, the linkage was stable in hydroxylamine, but sensitive to Hg²⁺, whereas the thiazolidine-type ADP-ribosylcysteine linkage is sensitive to both hydroxylamine and Hg²⁺ (26).
A different modification is the NAD-dependent covalent modification of glyceraldehyde-3-phosphate dehydrogenase that is stimulated by NO (15–22). The modification is not auto-ADP-ribosylation, but is in fact a novel linkage of intact NAD to a cysteine in ALDH, and was too limited to account for the much greater extent of enzyme inhibition (15–18, 22). Both ALDH and glyceraldehyde-3-phosphate dehydrogenase by NAD was also inhibited by the NO-independent modification with ADP-ribose, which was at stoichiometric levels and also probably at the active site.

Together, these nonenzymatic reactions of NAD or its metabolite ADP-ribose with thiols add a previously unrecognized level of complexity to the investigation of endogenous cysteine-specific ADP-ribosylation in mammalian cells. The one reported eukaryotic NAD:cysteine ADP-ribosyltransferase was purified on the basis of its ability to ADP-ribosylate cysteine methyl ester, without positive identification of a thioglycoside linkage in the product (34). As shown previously, the product of this enzyme could result from generation of ADP-ribose with a subsequent nonenzymatic condensation of ADP-ribose with cysteine methyl ester to yield a thiazolidine (26). Despite the uncertainty about endogenous NAD:cysteine ADP-ribosyltransferases, there is, nevertheless, direct evidence for the presence of ADP-ribose attached to cysteine residues in the plasma membrane fraction of rat liver (35). Circumstantial evidence consistent with the existence of ADP-ribosylcysteine linkages in animal cells comes from the identification of thioglycosidases in animals (36), including an enzyme that removes ADP-ribose from a cysteine in the α-subunit of the heterotrimeric guanine nucleotide-binding protein, Gα ("inhibitory" G-protein) (37).

The data reported here demonstrating high-stoichiometry nonenzymatic ADP-ribosylation of cysteine in ALDH, and the earlier results of nonenzymatic ADP-ribosylation of free cysteine (26), point out the precautions that must be taken in attempting to study endogenous ADP-ribosylation of cysteine residues.

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