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Nitric Oxide-Induced Modification of Glyceraldehyde-3-Phosphate Dehydrogenase with NAD^+ Is Not ADP-Ribosylation[1]

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One biological effect of nitric oxide (NO) has been believed to be exerted through induction of the ADP-ribosyltransferase activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Though this notion is based on the finding that NO increases the auto-ADP-ribosylation of GAPDH, controversial data have also been reported. To determine whether or not NO really activates ADP-ribosylation, we re-examined the NO-induced modification of GAPDH with NAD^+. GAPDH was modified equally with [adenosine-^{14}C]NAD^+ and [carbonyl-^{14}C]NAD^+, indicating that the glycoside bond of NAD^+ between ADP-ribose and nicotinamide is intact. The release of nicotinamide from NAD^+ was not evident during incubation of GAPDH with [carbonyl-^{14}C]NAD^+. Thus, the modification of GAPDH is apparently not ADP-ribosylation. In addition, we found that basal and glyceraldehyde-3-phosphate-induced modifications of GAPDH, both of which have also been explained as ADP-ribosylation, were not ADP-ribosylation, and that the modification of GAPDH in the absence and presence of NO or GA3P was distinct in the dithiothreitol effect or resistance to $HgCl_2$.

Key words: ADP-ribosylation, bovine brain, glyceraldehyde-3-phosphate, glyceraldehyde-3-phosphate dehydrogenase, nitric oxide.

NO mediates biological phenomena including blood vessel relaxation, cytotoxicity of macrophages, and neurotransmission (*1-4*). The well-known cellular target molecule of NO is guanylyl cyclase, which is activated by the gaseous mediator and produces cGMP (*5*). Recent work suggested the presence of a cGMP-independent pathway mediated by NO and that one of the candidate targets of NO in the cGMP-independent pathway is GAPDH, a key enzyme in glycolysis (*6-8*). The hypothesis that GAPDH is the target protein of NO is essentially based on the finding of NO-stimulated modification of GAPDH with NAD^+, which is considered by some workers to be "ADP-ribosylation" (*6-8*).

ADP-ribosylation is one of the post-translational covalent modifications of proteins and is catalyzed by ADP-ribosyltransferase. ADP-ribosyltransferase transfers an ADP-ribose moiety to specific amino acid residues, and releases nicotinamide from its substrate, NAD^+. Several ADP-ribosyltransferases specific to different amino acid residues have been isolated from bacterial and eucaryotic sources (*9*). The physiological significance of ADP-ribosylation is poorly understood and progress in this area has been impeded partly by the difficulty in identifying ADP-ribosylation reactions among different types of modifications involving NAD^+, including the non-enzymatic glycation of proteins by ADP-ribose produced by NAD^+ glycohydrolase during labeling (*10*).

In early works on NO-induced modification with [adenylate-^{32}P]NAD^+ of 36 to 41 kDa proteins in various tissues, ^{32}P-labeling of these proteins was referred to as "ADP-ribosylation," and further studies were not performed to confirm that the radiolabeling of these proteins was indeed the result of ADP-ribosylation by an NO-stimulated ADP-ribosyltransferase (*11-14*). Subsequent studies suggested the possibility that at least one of these proteins modified with [adenylate-^{32}P]NAD^+ in the presence of NO was GAPDH, and revealed that NO-induced modification of GAPDH with [adenylate-^{32}P]NAD^+ occurred even in a reconstitutional system which did not contain enzyme or protein components other than GAPDH itself (*6-8*). Moreover, GAPDH was reported to be labeled with [adenosine-^{14}C]NAD^+ but not with [carbonyl-^{14}C]NAD^+, thereby suggesting the release of the nicotinamide moiety of NAD^+ and the transfer of ADP-ribose to the protein (*7, 15*). These results taken together led to the hypothesis that GAPDH has ADP-ribosyltransferase activity and that it catalyzes auto-ADP-ribosylation. However, direct evidence of nicotinamide release has not been obtained. Using [adenosine- or carbonyl-^{14}C]NAD^+, McDonald and Moss obtained the opposite result, *i.e.* that both forms of labeled

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GA3P, glyceraldehyde-3-phosphate; IAP, islet-activating protein; KPB, potassium phosphate buffer; MSH, 2-mercaptoethanol; NO, nitric oxide; NOR1, (±)-(*E*)-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamine; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; SNP, sodium nitroprusside; TFA, trifluoroacetic acid.

NAD^+ equally modified GAPDH (*16*).

GAPDH catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate to form 1,3-diphosphoglycerate in the presence of NAD^+ and inorganic phosphate. Kots *et al.* showed the radiolabeling of GAPDH on incubation with [adenylate-^{32}P]NAD^+, that was induced by D-glyceraldehyde-3-phosphate, and this radiolabeling was inhibited in the presence of NO. This novel modification of GAPDH was also explained as ADP-ribosylation (*17*), but they did not determine whether or not this modification was actually ADP-ribosylation.

To better understand the mechanisms underlying NO-induced biological functions, it is crucial to determine whether or not GAPDH is an ADP-ribosyltransferase. Therefore, we addressed the question of whether or not GAPDH has ADP-ribosyltransferase activity. We report here evidence that GAPDH is not an ADP-ribosyltransferase catalyzing auto-ADP-ribosylation. Our results parallel those of McDonald and Moss (*16*), and raise doubt as to whether NO has stimulatory effects on ADP-ribosylation reactions.

EXPERIMENTAL PROCEDURES

Materials—[Adenylate-^{32}P]NAD^+ (29.6 TBq/mmol), [adenosine-^{14}C]NAD^+ (20.0 GBq/mmol), and EN^{3}HANCE were purchased from Du Pont-New England Nuclear. [Carbonyl-^{14}C]NAD^+ (2.0 GBq/mmol) was from Amersham. Rabbit muscle GAPDH and GA3P were from Sigma. NOR1 and SNAP were from Wako, Osaka.

Preparation of [^{32}P]ADP-Ribose—[Adenylate-^{32}P]-NAD^+ incubated with 25 mM NaOH at 37°C for 30 min was mixed with the same amount of 1% TFA, and then subjected to reverse phase HPLC on a Cosmosil 5C_{18}-AR column (Nacalai Tesque) at the flow rate of 0.5 ml per min of 0.1% TFA. The UV absorbance of the eluate was monitored at 254 nm. Under the chromatographic conditions used, the retention times of authentic ADP-ribose, 5′-AMP, nicotinamide, and NAD^+ were 6.6, 8.8, 12.2, and 22.7 min, respectively. The eluate was collected 0.5 ml per tube, and the radioactivity of an aliquot of each fraction was counted. The fractions corresponding to ADP-ribose were pooled and lyophilized to remove TFA. The purity of [^{32}P]ADP-ribose thus obtained was confirmed by HPLC on a C_{18} reverse phase column or a SAX anion exchange column (Waters).

Modification of GAPDH with Radiolabeled NAD^+s—GAPDH (3 μg) was incubated in a 40 μl reaction mixture comprising 50 mM Tris-HCl, pH 9.0, 5 mM DTT, 5 μM (185 kBq/nmol) [^{32}P]NAD^+ or [^{32}P]ADP-ribose, or 125 μM (2 kBq/nmol) [adenosine-^{14}C]NAD^+ or [carbonyl-^{14}C]-NAD^+ in the presence of 50 μM sodium nitroprusside (SNP) or 0.5 mM GA3P. After incubation at 37°C for 1 h, the reaction was terminated by the addition of 20 μl of a SDS-PAGE sample buffer (3% SDS, 3% MSH, 30 mM Tris-HCl, pH 6.8, and 60% glycerol), and then the sample (50 μl) was electrophoresed in a 12.5% polyacrylamide gel by the method of Laemmli (*18*). The gels were stained with Coomassie Brilliant Blue R-250 and then destained. For autoradiography of ^{32}P, the gel was dried and then exposed to a standard X-ray film for 12-24 h. To determine the incorporated radioactivity in individual proteins, the activity was quantified by use of an AMBIS 4000 (AMBIS Image Acquisition & Analysis). In experiments with ^{14}C-NAD^+, the destained gels were treated with EN^{3}HANCE according to the supplier's manual and then exposed to Hyperfilm-ECL (Amersham).

Release of Radioactivity from ^{32}P-Labeled GAPDH—^{32}P-labeled GAPDH was precipitated with 10% trichloroacetic acid. The pellet was washed twice with cold acetone, resuspended in 50 mM HEPES, pH 7.5, and 1% SDS containing 0.5 M NaCl, 0.5 M neutral hydroxylamine, or 50 μM $HgCl_2$, and incubated at 37°C for 1 h (*19*). The radioactivity of the IAP substrate modified with pertussis toxin was confirmed to be released on incubation with 50 μM $HgCl_2$ at 37°C for 1 h. After treatment with the reagents described above, the enzyme was reprecipitated with 10% trichloroacetic acid, and the pellet was dissolved in the SDS-PAGE sample buffer and then electrophoresed in a 12.5% polyacrylamide gel.

Measurement of GAPDH Activity—The assay mixture (2 ml), comprising 50 mM Tris-HCl, pH 8.5, 1 mM DTT, 250 μM NAD^+, 20 mM disodium hydrogen phosphate, and 0.6 mM GA3P, was kept at 25°C for 5 min in a spectrophotometer to achieve temperature equilibrium and to establish a blank value. The reaction was initiated by the addition of 1.5 μg of GAPDH to the mixture. The activity of GAPDH was determined by measuring the increase in absorbance at 340 nm of the reaction product, NADH.

Assay for Nicotinamide Release—The reaction mixture comprised 50 mM Tris-HCl, pH 9.0, and 13.9 μM [carbonyl-^{14}C]NAD^+ (2 kBq/nmol) or further addition of 50 μM SNP or 0.5 mM GA3P in the presence of 5 mM DTT in a total volume of 0.2 ml. The reaction was initiated by the addition of 15 μg of GAPDH, followed by incubation for 2 h at 37°C. For the control assay, the bovine brain soluble fraction (200 μg) served as the NAD^+ glycohydrolase and the reaction was started by adding it to a reaction mixture comprising 100 mM KPB, pH 7.5, and 13.9 μM [carbonyl-^{14}C]NAD^+ (2 kBq/nmol, 330,000 dpm/tube). The reaction was terminated by the addition of 0.2 ml of 5 N KCN and 0.4 ml of water-saturated ethyl acetate, and after standing for 15 min, the assay tubes were centrifuged for 10 min at 5,000 × *g*. The amount of radioactivity remaining in the ethyl acetate fraction after the centrifugation was measured (*20*).

Preparation of a Soluble Fraction from Bovine Brain—Bovine brain tissue (4 g) was homogenized in 4 ml of a homogenizing buffer comprising 250 mM sucrose, 5 mM Tris-HCl, pH 8.0, 3 mM $CaCl_2$, 1 mM EDTA, and 0.5 mM EGTA, by 4 strokes in a Teflon homogenizer at 10,000 rpm. The homogenate was centrifuged at 10,000 × *g* for 15 min. The supernatant was recentrifuged at 105,000 × *g* for 1 h. The final supernatant (10 mg/ml) was used as the "soluble fraction."

RESULTS

Different Types of Modification of GAPDH with [Adenylate-^{32}P]NAD^+—Figure 1 shows the autoradiographic analysis of the modification of GAPDH with [adenylate-^{32}P]NAD^+. An NO^+-donor, SNP, increased the incorporation of the radioactivity from [adenylate-^{32}P]NAD^+ into GAPDH in a DTT-dependent manner, as noted by other workers (*15*). When GAPDH was incubated with the labeled NAD^+, even in the absence of SNP or GA3P, the enzyme was labeled. The basal incorporation of radio-

activity into GAPDH (in contrast to NO- or GA3P-induced modification) was markedly inhibited in the presence of DTT. On the other hand, DTT increased the modification of GAPDH with NAD^+ in the presence of SNP or GA3P, while in the absence of an SH agent, these two compounds enhanced the labeling of GAPDH to a small extent. The addition of both SNP and DTT increased the radiolabeling of GAPDH to about 5-fold the basal activity, but the combination of DTT and GA3P was most effective in enhancing the labeling of GAPDH, to approximately 20-fold the basal radioactivity, while the addition of SNP inhibited the incorporation achieved with the combination of GA3P and DTT. Another co-substrate of GAPDH, inorganic phosphate, however, had no effect on the modification of GAPDH with [^{32}P]NAD^+ at all (data not shown).

The chemical stability of these covalent modifications was then examined (Fig. 2). The SNP-induced modification was sensitive to $HgCl_2$ treatment, implying the involvement of an SH-group, namely a cysteine residue (*19*). The GA3P-dependent modification was resistant to both neutral NH_2OH and $HgCl_2$ treatment. These results were consistent with documented data (*7, 15, 17*). Moreover, the basal incorporation, which was thought to be activated on the addition of SNP (*15*), was resistant as to $HgCl_2$ treatment, indicating that the modification differs from that in the case of SNP. The modifications occurring under three different conditions (in the absence or presence of SNP or GA3P) were not identical, at least in terms of the chemical stability as to $HgCl_2$ treatment and the effect of DTT.

Possibility of ADP-Ribosylation—There is the possibility that modifications of GAPDH with NAD^+ represent non-enzymatic covalent binding of labeled ADP-ribose contaminating [adenylate-^{32}P]NAD^+ or produced from NAD^+ during the incubation, since ADP-ribose has been reported to bind to proteins covalently *via* Schiff base formation or other chemical bonds (*10*). The labeling of GAPDH induced by GA3P was explained by Schiff base formation between ADP-ribose and the amino group of GAPDH (*17*). If this is the case, the unlabeled ADP-ribose should inhibit the labeling of GAPDH with [^{32}P]NAD^+. The addition of unlabeled ADP-ribose to the labeling mixture, however, did not decrease the labeling of GAPDH, while the addition of unlabeled NAD^+ effectively suppressed the modification (data not shown). Furthermore, the incorporation of radioactivity from [^{32}P]ADP-ribose into GAPDH was much lower than that from [^{32}P]NAD^+ with the same specific activity, in all cases (Fig. 3), indicating that the non-enzymatic modification of GAPDH with [^{32}P]ADP-ribose derived from NAD^+ is unlikely.

There is also the possibility that GAPDH itself has ADP-ribosyltransferase activity which catalyzes the cleavage of the *N*-glycosidic bond between ADP-ribose and nicotinamide in NAD^+, and the transfer of ADP-ribose to its own amino acid residues, as stated in the literature (*7, 15, 21*). To determine whether or not GAPDH has ADP-ribosyltransferase activity, we measured [^{14}C]nicotinamide release during the incubation of GAPDH with [carbonyl-^{14}C]NAD^+ (Table I). The release of nicotinamide from NAD^+ did not increase in the presence of GAPDH, compared to that in the absence of GAPDH. Furthermore,

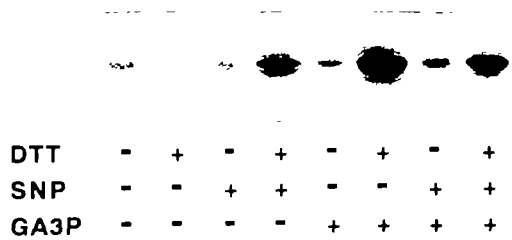

Fig. 1. **Radiolabeling of GAPDH with [adenylate-^{32}P]NAD^+ in the presence of combinations of various agents.** GAPDH was incubated with 5 μM (185 kBq/nmol) [adenylate-^{32}P]NAD^+ in the presence of the indicated agents, and then analyzed by SDS-PAGE followed by autoradiography. The concentrations of DTT, SNP, and GA3P in the reaction mixtures were 5 mM, 50 μM, and 0.5 mM, respectively.

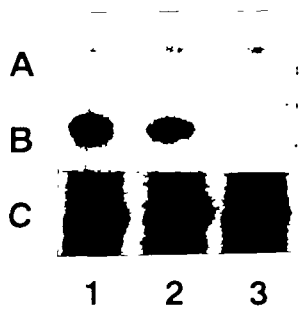

Fig. 2. **Release of radioactivity from ^{32}P-labeled GAPDH.** GAPDH was incubated with 50 mM Tris-HCl, pH 9.0, and 5 μM (185 kBq/nmol) [adenylate-^{32}P]NAD^+. The additions were none (A), 5 mM DTT and 50 μM SNP (B), and 5 mM DTT and 0.5 mM GA3P (C). The labeled GAPDHs were treated with 0.5 M NaCl (lane 1), 0.5 M NH_2OH (lane 2), or 50 μM $HgCl_2$ (lane 3), and then analyzed as described under "EXPERIMENTAL PROCEDURES."

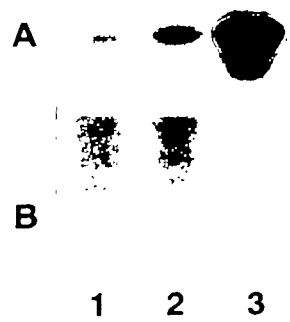

Fig. 3. **Labeling of GAPDH with [adenylate-^{32}P]NAD^+ or [adenylate-^{32}P]ADP-ribose.** GAPDH was incubated with 5 μM (185 kBq/nmol) [^{32}P]NAD^+ (A) or [^{32}P]ADP-ribose (B), and then analyzed by SDS-PAGE followed by autoradiography. The additions to the labeling mixture were none (lane 1), 50 μM SNP (lane 2), and 0.5 mM GA3P (lane 3).

TABLE I. **Measurement of [^{14}C]nicotinamide released during the incubation of GAPDH with [carbonyl-^{14}C]NAD^+.**

Additions	[^{14}C]Nicotinamide released (dpm/min)
None	22.4
GAPDH	22.2
GAPDH+SNP	21.2
GAPDH+GA3P	20.4
Bovine brain soluble fraction	401.2

the addition of SNP or GA3P did not increase the release of nicotinamide. These results do not support the notion that GAPDH is an ADP-ribosyltransferase or that the modification is the auto-ADP-ribosylation described in the literature (*7, 8, 15, 21*).

As free nicotinamide was not detected during the labeling reaction, we examined whether or not the nicotinamide group was retained in the modified GAPDH. GAPDH was incubated with [adenosine-^{14}C]NAD$^+$ or [carbonyl-^{14}C]-NAD$^+$, and then the labeling was monitored by fluorography after SDS-PAGE (Fig. 4). This same experiment has been performed in three different laboratories. Kots *et al.* (*7*) and Dimmeler *et al.* (*6*) reported that GAPDH was only modified with [adenosine-^{14}C]NAD$^+$, *i.e.* it was not modified with [carbonyl-^{14}C]NAD$^+$. On the other hand, McDonald and Moss (*16*) found that GAPDH was modified equally with [adenosine-^{14}C]NAD$^+$ and [carbonyl-^{14}C]-NAD$^+$. In our experiments, GAPDH was modified with [adenosine-^{14}C]NAD$^+$ and [carbonyl-^{14}C]NAD$^+$ to similar extents under all conditions examined. As [carbonyl-^{14}C]-NAD$^+$ can label GAPDH, the possibility that the modification comprises the transfer of ADP-ribose from NAD$^+$ can be excluded. McDonald and Moss reported that products released by Hg^{2+} from GAPDH labeled with [adenosine-^{14}C]NAD$^+$ or [carbonyl-^{14}C]NAD$^+$ exhibited the same retention times on HPLC analysis (*16*), a result which shows that in the case of SNP-induced modification, the linkage between ADP-ribose and nicotinamide was intact even after release from the protein.

Does GAPDH Modified with NAD$^+$ in the Presence of GA3P Function as an Intermediate in the Catalytic Reaction?—Since NAD$^+$ is a substrate for GAPDH, which catalyzes the oxidative phosphorylation of GA3P, the possibility that NAD$^+$-labeled GAPDH in the presence of GA3P is an intermediate of the reaction was examined by means of pulse chase experiments (Fig. 5). GAPDH labeled with [^{32}P]NAD$^+$ in the presence of GA3P was incubated further with a large amount of unlabeled NAD$^+$, P_i, and additional GA3P, components required for GAPDH activity. If the labeled protein serves as an intermediate of the reaction, the radioactivity should be released with the progress of the reaction. Even after 2 h of the chase, there was no decrease in the labeling of the protein. This phenomenon was not due to the inactivation of GAPDH, since 68% of the enzyme activity remained at the start of the chase (Fig. 6). Thus, the modification apparently does not play a role in the dehydrogenase reaction.

Alteration of GAPDH Activity on Treatment with SNP or GA3P—Next we examined the dehydrogenase activity of GAPDH with each type of modification (Fig. 6). When GAPDH was incubated with SNP for 2 h, its dehydrogenase

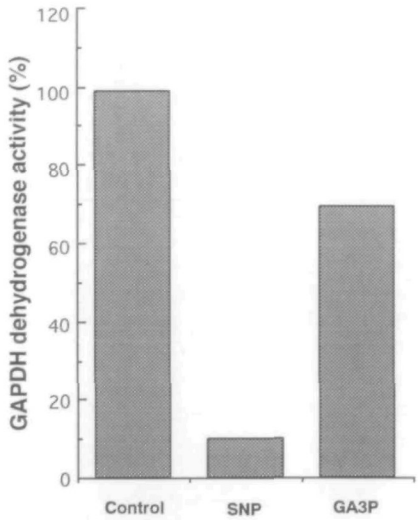

Fig. 6. **Inhibition of GAPDH activity by SNP- or GA3P-induced modification.** GAPDH was incubated in the presence of 50 μM SNP or 0.5 mM GA3P for 2 h, and the dehydrogenase activity was measured as described under "EXPERIMENTAL PROCEDURES." The activity of GAPDH incubated with no addition for 2 h was taken as 100%.

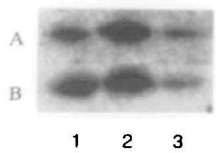

Fig. 4. **Labeling of GAPDH with [adenosine-^{14}C]NAD$^+$ or [carbonyl-^{14}C]NAD$^+$.** GAPDH was incubated with 125 μM (2 kBq/nmol) [adenosine-^{14}C]NAD$^+$ (A) or [carbonyl-^{14}C]NAD$^+$ (B) in the presence of 50 μM SNP (lane 1), 0.5 mM GA3P (lane 2), or neither (lane 3), and then analyzed by SDS-PAGE followed by autoradiography.

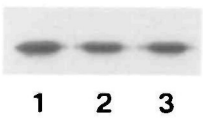

Fig. 5. **Comparison of the radioactivity incorporated into GAPDH in pulse chase experiments.** GAPDH was labeled with 5 μM (185 kBq/nmol) [adenylate-^{32}P]NAD$^+$ in the presence of 0.5 mM GA3P for 2 h (lane 1), and then added to the GAPDH assay mixture containing unlabeled NAD$^+$ (lane 2), and incubated for a further 2 h (lane 3), followed by SDS-PAGE analysis. The final concentrations of NAD$^+$, GA3P, and P_i were 0.5, 2, and 40 mM, respectively.

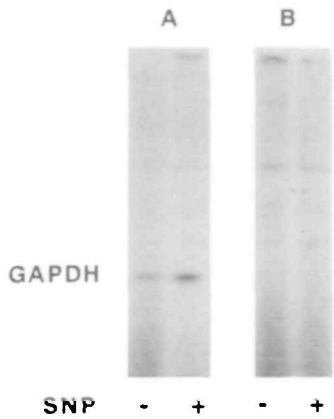

Fig. 7. **Labeling of several proteins in the bovine brain soluble fraction with [adenylate-^{32}P]NAD$^+$ or [adenylate-^{32}P]ADP-ribose in the presence or absence of SNP.** The bovine brain soluble fraction (10 μl) was incubated in a 40 μl reaction mixture comprising 50 mM Tris-HCl, pH 9.0, 2.5 mM DTT, and 5 μM (185 kBq/nmol) [^{32}P]NAD$^+$ (A) or [^{32}P]ADP-ribose (B) in the presence or absence of 50 μM SNP, and then analyzed by SDS-PAGE followed by autoradiography.

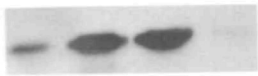

Fig. 8. **Labeling of GAPDH with [adenylate-^{32}P]NAD$^+$ by SNAP, SNP, or NOR1.** GAPDH was incubated with 250 μM SNAP (lane 1), 50 μM SNP (lane 2), 50 μM NOR1 (lane 3), or no addition (lane 4) in a reaction mixture comprising 50 mM Tris-HCl, pH 9.0, 5 mM DTT, and 5 μM (185 kBq/nmol) [adenylate-^{32}P]NAD$^+$, and then analyzed by SDS-PAGE followed by autoradiography.

activity decreased by approximately 90%. Under the same conditions, when it was treated with GA3P, its enzyme activity remained at about 70% of the control level. Although radiolabeling of GAPDH modified with GA3P was at least 5-fold higher than that of the SNP-modified GAPDH, modification with GA3P was less effective than with SNP as to dehydrogenase activity.

Does ADP-Ribosylation by GAPDH Occur in the Brain?—As described above, GAPDH was never auto-ADP-ribosylated in the presence of SNP or GA3P. However, the possibility remained that GAPDH can ADP-ribosylate other proteins. Thus, we examined this possibility in a bovine brain soluble fraction, in which GAPDH is abundantly present. Since the bovine brain soluble fraction contained NAD$^+$ glycohydrolase (*22*), NAD$^+$ in the assay mixture was readily cleaved into nicotinamide and ADP-ribose. Therefore, the bovine brain soluble fraction was incubated with [adenylate-^{32}P]NAD$^+$ or its breakdown product, [adenylate-^{32}P]ADP-ribose, in the presence or absence of SNP, and proteins radiolabeled by these nucleotides were compared by SDS-PAGE autoradiographical analysis (Fig. 7). The 39 kDa protein, the labeling of which was enhanced by SNP, was identified as GAPDH on sequencing analysis (data not shown). The radiolabeling of GAPDH was only enhanced in the presence of SNP in the case of incubation with [adenylate-^{32}P]NAD$^+$, and it was not radiolabeled in the presence or absence of SNP in the case of incubation with [adenylate-^{32}P]ADP-ribose. In both cases, the radiolabeling of other proteins was not affected in either the presence or absence of SNP. These results suggest that GAPDH has no potential to ADP-ribosylate other proteins, at least in the assay system we used.

Effect of NOR1 or SNAP on the Modification of GAPDH—We also investigated the effect of NOR1, a pure NO-releasing agent, and SNAP, a nitrosothiol, on this modification (Fig. 8). NOR1 was as effective as to the incorporation of radioactivity into GAPDH as compared SNP. SNAP was also efficacious but to a lesser extent. These results exclude the possibility that the CN$^-$ and iron ions present in SNP are involved in the modification of GAPDH with NAD$^+$.

DISCUSSION

The purpose of this study was to determine whether or not GAPDH is an ADP-ribosyltransferase. Since several different types of non-enzymatic modifications of amino acids by ADP-ribose have been reported (*10*), we first ruled out the possibility that the labeling of GAPDH with [^{32}P]NAD$^+$ comprises non-enzymatic glycation of the protein by a trace amount of ADP-ribose, present in the NAD$^+$ preparation or generated during the labeling reaction, by the observation that [^{32}P]NAD$^+$ but not [^{32}P]ADP-ribose labeled GAPDH, and that unlabeled ADP-ribose did not decrease the GAPDH modification with [^{32}P]NAD$^+$.

If GAPDH is an ADP-ribosyltransferase, the enzyme would release nicotinamide from NAD$^+$ during the transferase reaction, thus the nicotinamide moiety would not exist in the ADP-ribosylated protein. In our experiments, however, GAPDH did not produce free [^{14}C]nicotinamide on incubation with [carbonyl-^{14}C]NAD$^+$ and, furthermore, [carbonyl-^{14}C]NAD$^+$ labeled GAPDH to a similar extent to that obtained with [adenylate-^{14}C]NAD$^+$. This latter result is consistent with that obtained by McDonald and Moss (*16*), but contrary to data of Kots *et al.* (*7*) and Dimmeler and Brüne (*15*). Since the latter two groups did not document the precise details, the reason for the differences between their data and those of McDonald and Moss (*16*) cannot be determined.

Here, we confirmed that the labeling of GAPDH with radioactive NAD$^+$ is not due to ADP-ribosylation. Thus, the hypothesis that GAPDH is an ADP-ribosyltransferase does not have strong support. Despite these conflicting results, at least one possibility has to be considered. Following reports of NO-activated "ADP-ribosyltransferase" (*7, 15, 21*), several investigators found an NO-induced increase in the labeling of proteins other than GAPDH with radioactive NAD$^+$, and explained it as a result of the activation of ADP-ribosyltransferase by NO (*12, 14, 23-25*). Thus, one could argue that GAPDH can ADP-ribosylate other proteins, though it does not ADP-ribosylate itself. We examined the labeling of brain cytosolic proteins with NAD$^+$ and the effect of NO on the labeling. At first, we again tried to rule out the non-enzymatic ADP-ribosylation of proteins. Since NAD$^+$ glycohydrolase activity is abundant in the brain (*22*), it is important to detect enzymatic ADP-ribosylation to demonstrate that labeling did not occur with [^{32}P]ADP-ribose. Practically, all the labeling, except that of GAPDH, seen on incubation with [^{32}P]NAD$^+$ was also found with [^{32}P]ADP-ribose, thereby indicating that these are not cases of enzymatic ADP-ribosylation. Even after the addition of SNP, the labeling occurring only with [^{32}P]NAD$^+$ was not detected with other than GAPDH. Thus, at least under our conditions, no ADP-ribosyltransferase activated by NO was detected. Our data also showed that the detection of several radiolabeled proteins after incubation with radioactive NAD$^+$ does not directly indicate the presence of enzymatic ADP-ribosylation, especially in crude samples such as brain cytosol. For verification, one needs to show that labeling occurs with NAD$^+$ but not with ADP-ribose.

The labeling with [carbonyl-^{14}C]NAD$^+$ indicates that the modification of GAPDH with NAD$^+$ is not ADP-ribosylation, but rather covalent binding of NAD$^+$ itself or its nitrosyl derivatives. McDonald and Moss proposed that the nitrosyl derivative of NAD$^+$ bound to GAPDH in the case of NO-induced modification (*16*).

Mohr *et al.* suggested the importance of *S*-nitrosylation of the active site thiol of GAPDH by NO$^+$ for modification of the protein with NAD$^+$ (*26*). In this study, however, NOR1, an ·NO-releasing agent, was more effective for the modification of GAPDH than SNAP. The discrepancy may be due to the different reaction conditions.

The labeling of GAPDH with NAD$^+$, however, occurs even in the absence of an NO$^+$-donor, as we described in

this report, and these modifications (occurring with no addition, or with SNP or with GA3P) are different in their properties. Since GA3P and NAD^+ are substrates of GAPDH, we examined the possibility that the labeling with NAD^+ induced by GA3P represents the presence of an intermediate of the GAPDH reaction. The pulse-chase experiment on GAPDH with NAD^+ showed no marked decrease in the incorporated radioactivity on further incubation with a large amount of unlabeled NAD^+, thereby excluding intermediate formation. The extent of the modification of GAPDH with GA3P was much greater than that with NO. The modification with GA3P, in terms of physiological events, awaits further studies.

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