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Mechanism of covalent modification of glyceraldehyde-3-phosphate dehydrogenase at its active site thiol by nitric oxide, peroxynitrite and related nitrosating agents

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Abstract

Previous studies have suggested that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) undergoes covalent modification of an active site thiol by a NO•-induced [^{32}P]NAD$^+$-dependent mechanism. However, the efficacy of GAPDH modification induced by various NO donors was found to be independent of spontaneous rates of NO• release. To further test the validity of this mechanism, we studied the effects of nitrosonium tertrafluoroborate (BF_4NO), a strong NO^+ donor. BF_4NO potently induces GAPDH labeling by the radioactive nucleotide. In this case, the addition of thiol significantly attenuates enzyme modification by competing for the NO moiety in the formation of RS–NO. Peroxynitrite ($ONOO^-$) also induces GAPDH modification in the presence of thiol, consistent with the notion that this species can transfer NO^+ (or NO_2^+) through the intermediacy of RS–NO. However, the efficiency of this reaction is limited by $ONOO^-$-induced oxidation of protein SH groups at the active site. $ONOO^-$ generation appears to account for the modification of GAPDH by SIN-1. Thus, S-nitrosylation of the active site thiol is a prequisite for subsequent post-translational modification with NAD^+, and emphasizes the role of NO^+ transfer in the initial step of this pathway. Our findings thus provide a uniform mechanism by which nitric oxide and related NO donors initiate non-enzymatic ADP-ribosylation (like) reactions. In biological systems, endogenous RS–NO are likely to support the NO group transfer to thiol-containing proteins.

Key words: Nitric oxide; Thiol modification; GAPDH; S-Nitrosylation

1. Introduction

Nitric oxide (NO•) has gained wide attention because of its role in diverse biological processes (see [1,2] for references). The molecule is synthesized by a family of enzymes termed NO• synthases (NOS) which utilize arginine as their substrate in the 5-electron oxidation of the guanidino nitrogen to NO. However, once produced by the actions of NOS, NO• is extremely susceptible to both oxidation and reduction, resulting in the formation of surrogates that retain NO-like bioactivity [3]. Hence, the species accountable for the biological response is often ambiguous. This point is well exemplified in the case of *S*-nitrosothiols (RS–NO) which are found in many biological systems, and among their diverse function serve as bioactive reservoirs of NO that target reactive sulfhydryl centers [4]. In fact, such heterolytic pathways of RS–NO decomposition (i.. NO^+ transfer) may predominate in many biological systems and account for activity. *S*-Nitrosothiol–thiol exchange reactions are representative of such heterolytic mechanisms of RS-NO decomposition and have been invoked in the allosteric modulation of NMDA receptors to limit neurotoxicity [5], the antimicrobial effects of RS-NO [6], the inhibition of sulfhydryl containing enzymes [7,8] and the mechanism of activiation of stable protein RS–NO in vivo [3].

The signal transduction pathways of NO• can be broadly classified as either cyclic GMP-dependent or -independent: the former pathway is reasonably well elucidated and mediates the actions of NO• in smooth muscle and platelets (see [1,2] for references). Conformational changes induced by NO binding at the heme active site of soluble guanylyl cyclase lead to enzymatic conversion of guanosine triphosphate to guanosine cyclic 3′,5′-monophosphate. The cyclic GMP-independent mechanisms, however, are less well understood. In this regard, S-nitrosylation of proteins [9], and ADP-ribosylation (like) reactions [10] have received significant attention. *S*-Nitrosylated peptides and proteins have been identified in the salivary gland of rhodnius prolixus [11], human plasma [3], airway lining fluid [12] and neutrophils [13]. Studies on the mechanism of ADP-ribosylation have centered on the covalent NAD^+-dependent modification of the glycolytic enzyme [14], glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a likely function of its intracellular prevalence [15]. This NO-dependent reaction has been observed in a variety of different cellular systems on activation of NOS, or induced by the exogenous administration of NO-releasing compounds. Nevertheless, saturated gas NO solutions show weak activity, even in the presence of oxygen and transition metals (see [15] for references). Extending

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SNP, sodium nitroprusside; SIN-1, 3-morpholinosydnonimine; DTT, dithiothreitol; NAD^+, nicotinamide adenine dinucleotide.

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these observations, we observed that the efficacy of various NO donors at inducing GAPDH modification seemed independent of rates of NO• release (Table 1).

The present study was therefore undertaken to better characterize the mechanism by which NO• and related NO donors initiate ADP-ribosylation (like) reactions at sulfur-containing enzyme centers.

2. Materials and methods

2.1. Materials

[^{32}P]NAD$^+$ (800 Ci/mmol) was purchased from Du Pont-New England Nuclear. SIN-1 was provided by Cassella AG, Frankfurt, Germany. BF_4NO was bought from Aldrich, while BF_4NO_2 was obtained from Fluka. Rabbit muscle GAPDH (80 U/mg) was ordered from Boehringer-Mannheim. Diethylamine NO and spermine NO were obtained from NCI Chemical Carcinogen Reference Standard Repositories, Kansas City, USA. All other chemicals were purchased from Sigma at the highest grade of purity available.

2.2. Peroxynitrite synthesis

Peroxynitrite was synthesised as described previously [17]. Briefly, an ice-cold solution of 0.6 M HCl, 0.7 M H_2O_2 (10 ml) was added simultaneously to a well stirred, cooled (4°C) solution of 0.6 M $NaNO_2$ (10 ml), followed immediately by addition of 1.5 M NaOH (20 ml). Excess H_2O_2 was removed by addition of MnO_2. The mixture was shaken for 30 min at 4°C and then filtered (3 times; in order to remove MnO_2). This solution was frozen at −20°C for up to 1 week. Peroxynitrite forms a yellow top layer due to freeze fractionation, which was retained for further studies. The top layer typically contained 50–150 mM peroxynitrite as determined by UV-absorbance spectroscopy at 302 nm in 1 N NaOH ($\varepsilon_{302\,nm}$ = 1670 $M^{-1} \cdot cm^{-1}$) [17].

2.3. Covalent modification of GAPDH

GAPDH modifications were generally carried out as previously described (see [15] for references). GAPDH (10 μg/assay) was incubated in 100 mM HEPES buffer (pH 7.5), 0.15 μCi [^{32}P]NAD$^+$, 1–10 μM unlabeled NAD$^+$ in the absence or presence of 2.5 mM DTT, and a NO donor, respectively. After 30 min at 37°C, proteins were precipitated with 20% trichloroacetic acid followed by centrifugation (15 min at 13,000 × g, 4°C). The resulting pellets were washed twice with cold water-saturated ether and resolved in a 10% SDS-polyacrylamide gel. Radioactivity was quantified using the phosphor imager system (Molecular Dynamics).

2.4. Modification of GAPDH with nitrosonium-tetrafluoroborate and peroxynitrite

Stock solutions of BF_4NO and BF_4NO_2 were prepared under acidic conditions (pH 2; 0.2 M HCl), which promote stability of NO^+/NO_2^+. 1–2 μl were transferred to our complete GAPDH labeling assay, while carefully excluding any pH changes. Peroxynitrite ($ONOO^-$) was rapidly diluted into HEPES buffer maintaining an alkaline pH. For GAPDH modification, 1–10 μl were then transferred to our complete labeling assay (pH 7.4). BF_4NO and peroxynitrite solutions were prepared fresh every day, just prior to experiments.

2.5. Pre-incubation experiments using peroxynitrite

GAPDH was pre-incubated with different concentrations of peroxynitrite in the presence or absence of 2.5 mM DTT for 1 min at 37° C, followed by the addion of 200 μM SIN-1. Incubations were performed for an additional 30 min, essentially as described in Fig. 1.

3. Results and discussion

Previous studies have shown that the active site thiol of GAPDH is subject to NO-dependent modification by NAD$^+$. We further probed the chemical mechanism of the post-translational modification with the use of several NO donors. Sodium nitroprusside (SNP) was found to induce incorporation of radiolabeled nucleotide in the absence of added reducing equivalents. SNP contains an NO group with strong NO$^+$ character, and under certain conditions does not liberate NO• spontaneously [18]. These observations suggest that the NO$^+$ moiety is transferred to the critical thiol of GAPDH in the process of covalent modification. The addition of DTT was found to enhance SNP-induced labeling of the enzyme (Fig. 1). Various interpretations of this finding are compatible with a mechanism involving S-nitrosylation of active site thiol. First, DTT engenders a reducing environment that is a prequisite for any modification of the enzyme active site. Second, the reaction of SNP and thiol (i.e. DTT) is reported to yield RS–NO which has been shown to induce modification of GAPDH [14]. In this regard, the greater efficacy of RS–NO over SNP may be explained by steric constraints at the enzyme active site which limit access of the pentacyanoferrate moiety of SNP. Thiol has also been reported to facilitate the liberation of NO• from SNP, although this may occur, at least in part, by way of the intermediates of RS–NO (see [18] for references).

In order to better characterize the redox species involved in the mechanism of GAPDH modification, studies with the NO$^+$ donor nitrosonium tetrafluoroborate (BF_4NO) were performed. This compound was found to be a potent inducer of GAPDH modification. Addition of 1 or 2 μl of freshly prepared BF_4NO stock solutions to our GAPDH labeling assay system in the presence of [^{32}P]NAD$^+$ produces significant enzyme modification, at the relatively low concentrations of 50 μM. In contrast to SNP, however, the addition of DTT significantly attenuates GAPDH modification. Thus, the competing S-nitrosation reaction with DTT, present in high excess over enzyme, predictably hinders the reaction with the active site thiol. These data implicate NO$^+$ as the species transferred to the active site thiol in the mechanism of covalent modification of the enzyme. Our findings are in keeping with previous indirect evidence that protein

Table 1
Correlation between NO• generation and GAPDH modification

	Rates of NO• release ($t_{1/2}$)*	GAPDH modification by NAD$^+$
Diethylamine NO	2.2 min	+
Spermine NO	~ 40 min	+
S-NO-glutathione	7.8 hr	++
Sodium nitroprusside	ND	++
SIN-1	ND**	+++

GAPDH modification was assayed under standard conditions.
*Assayed by NO electrode (WPI electrode) and/or oxidation of oxymyoglobin to metmyoglobin [16]. $t_{1/2}$ based on the assumption of first order kinetics.
**Variable results, but generally undetectable or very little release of NO. ND, not detectable.

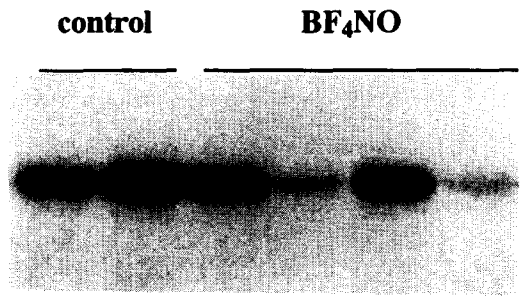

Fig. 1. Modification of GAPDH by nitrosonium tetrafluoroborate. Covalent GAPDH modification in the presence of [^{32}P]NAD$^+$ was carried out as described in section 2. Incubations were performed for 30 min with DTT, sodium nitroprusside (SNP), and BF_4NO. The figure is representative of four similar experiments.

S-nitrosylation preceeds [19], and thereby, initiates subsequent covalent NAD$^+$-dependent protein modification.

Studies with the NO donor SIN-1 have led to conflicting reports on the mechanism of GAPDH modification. This compound generates nitric oxide and superoxide stoichiometrically, at the same time. The diffusion-limited radical-radical interaction between these species leads to the formation of peroxynitrite ($ONOO^-$) [20]. In our studies, the addition of superoxide dismutase was found to inhibit SIN-1-induced labeling of the enzyme. Direct measurements of NO$^\bullet$ (WPI microelectrode) demonstrated that superoxide dismutase enhances NO$^\bullet$ production from SIN-1 under these conditions. Taken together, these data support the conclusion that peroxynitrite mediates the action of SIN-1. In order to reconcile these findings with a mechanism involving S-nitrosylation, we examined the effects of peroxynitrite in greater detail.

As illustrated in Fig. 2, peroxynitrite causes radioactive labeling of GAPDH, confirming previous reports [10]. However, the activity of $ONOO^-$ was found to be critically dependent on the presence of thiol, whereas addition of SOD had no effect. Peroxynitrite (50–100 μM) did not produce substantial protein modification unless DTT was included in the reaction mixtures. Under these conditions $ONOO^-$ was more potent than SNP. These findings can be well rationalized by the known reactions of peroxynitrite with thiol groups. When present in relative excess, peroxynitrite oxidizes thiols to sulfenic or sulfonic acids [21,22], thereby inhibiting subsequent covalent modification by NAD$^+$. This notion is supported by the finding that pretreatment of GAPDH with peroxynitrite attenuates the incorporation of radioactivity on subsequent stimulation with SIN-1 (Fig. 3).

Indeed, peroxynitrite-induced oxidative inactivation of other thiol-containing enzymes, such as succinate dehydrogenase, has recently been reported [23]. In contrast, when thiol is present in relative excess over peroxynitrite, conditions are conducive to the formation of RS–NO [8,24]. The sequence of events most likely involves S-nitrosation of DTT by $ONOO^-$ followed by NO$^+$ donation to the reactive protein SH group. The finding that superoxide generating systems (i.e. xanthine/xanthine oxidase) do not enhance SNP-induced covalent modification of GAPDH in systems replete in thiol (i.e. capable of generating NO$^\bullet$) ($n = 3$) is in keeping with our findings that peroxynitrite does not play a direct role in this pathway. Moreover, these same studies support the previous conclusion that nitric oxide itself is not directly involved, as superoxide enhances nitric oxide release from SNP [25].

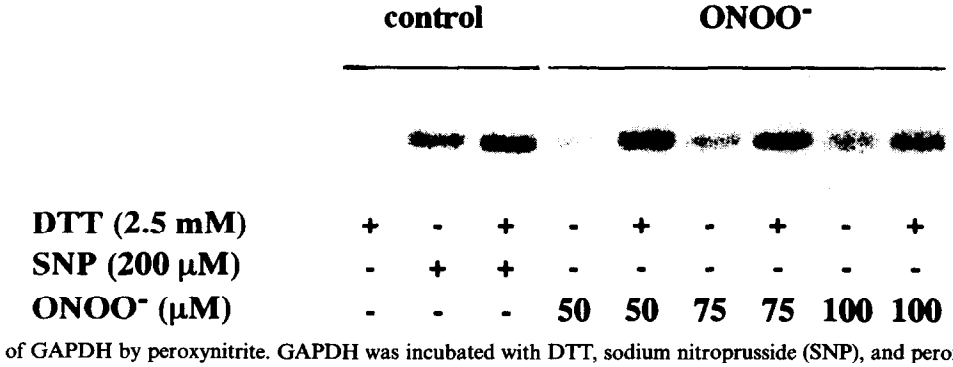

Fig. 2. Modification of GAPDH by peroxynitrite. GAPDH was incubated with DTT, sodium nitroprusside (SNP), and peroxynitrite. Other details are as described in Fig. 1 and section 2. Results are representative of eight similar assays.

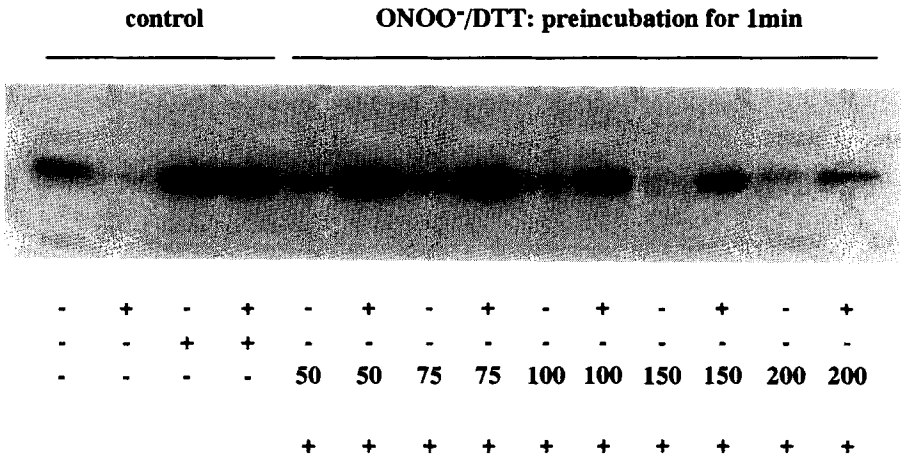

Fig. 3. Peroxynitrite modulates SIN-1 induced covalent GAPDH modification. GAPDH was incubated with DTT, SIN-1 and peroxynitrite as indicated. The preincubation of DTT/$ONOO^-$, followed by addition of SIN-1 was carried out as described in section 2. Other details are as in Fig. 1. Data are representative of three similar experiments.

Heterolytic mechanisms of $ONOO^-$ cleavage in physiological systems may result in nitration (i.e. reaction of transfer of NO_2^+) of nucleophilic centers [26,27]. As the intermediates of thionitrates (RS–NO_2) had not been excluded in studies in which peroxynitrite induced covalent modification of GAPDH, this possibility was further explored. Nitronium tetrafluoroborate (BF_4NO_2), a strong NO_2^+ donor, was found to be very effective at inducing covalent modification with NAD^+ in the absence of DTT. This activity was comparable to BF_4NO and was likewise inhibited by thiol. The physiological importance of this observation notwithstanding, S-nitration of the enzyme thus supports subsequent covalent modification with NAD^+. This has important mechanistic implications, as it indicates that chemistry shared by NO^+ and NO_2^+ initiates subsequent covalent modification with NAD^+.

Our findings provide a uniform mechanism by which nitric oxide and related NO donors initiate non-enzymatic ADP-ribosylation (like) reactions. S-Nitrosylation of the acitive site thiol is a prequisite for subsequent modification with NAD^+, and emphasizes the role of NO group transfer chemistry in the initial step of this mechanism. In biological systems, RS–NO most likely support the transfer of the NO moiety to reactive sulfhydryl centers of proteins.

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