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Nitric oxide-dependent NAD linkage to glyceraldehyde-3-phosphate dehydrogenase: possible involvement of a cysteine thiyl radical intermediate

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Previous studies have demonstrated that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) undergoes NAD(H) linkage to an active site thiol when it comes into contact with ·NO-related oxidants. We found that a free-radical generator 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH), which does not release either ·NO or ·NO-related species, was indeed able to induce the NAD(H) linkage to GAPDH. We performed spin-trapping studies with purified apo-GAPDH to identify a putative thiol intermediate produced by AAPH as well as by ·NO-related oxidants. As ·NO sources we used ·NO gas and two ·NO-donors, *S*-nitroso-*N*-acetyl-D,L-penicillamine and 3-morpholinosydnonimine hydrochloride (SIN-1). Because SIN-1 produces ·NO and a superoxide radical simultaneously, we also tested the effects of peroxynitrite. All the ·NO-related oxidants were able to induce the linkage of NAD(H) to GAPDH and the formation of a protein free-radical identified as a thiyl radical (inhibited by *N*-ethylmaleimide). ·NO gas and the ·NO-donors required molecular oxygen to induce the formation of the GAPDH thiyl radical, suggesting the possible involvement of higher nitrogen oxides. Thiyl radical formation was decreased by the reconstitution of GAPDH with NAD$^+$. Apo-GAPDH was a strong scavenger of AAPH radicals, but its scavenging ability was decreased when its cysteine residues were alkylated or when it was reconstituted with NAD$^+$. In addition, after treatment with AAPH, a thiyl radical of GAPDH was trapped at high enzyme concentrations. We suggest that the NAD(H) linkage to GAPDH is mediated by a thiyl radical intermediate not specific to ·NO or ·NO-related oxidants. The cysteine residue located at the active site of GAPDH (Cys-149) is oxidized by free radicals to a thiyl radical, which reacts with the neighbouring coenzyme to form Cys-NAD(H) linkages. Studies with the NAD$^+$ molecule radiolabelled in the nicotinamide or adenine portion revealed that both portions of the NAD$^+$ molecule are linked to GAPDH.

INTRODUCTION

Free radicals can be generated in normal cellular redox processes or through the interaction of cells and tissues with a variety of external agents [1]. Thiol groups, present either in cysteine residues of proteins or in low-molecular-mass compounds, are common targets for free radicals, with thiol oxidation being a key mechanism of free-radical-mediated toxicity at the molecular level [2].

Nitric oxide (·NO) is a biological messenger involved in multiple biological functions including vessel smooth-muscle relaxation, platelet aggregation, neurotransmission modulation and phagocyte cytotoxicity [3]. The intracellular targets of ·NO include haem proteins and proteins with iron–sulphur centres. Moreover, ·NO in the presence of oxygen is transformed into a nitrosating species that reacts with both low- and high-molecular-mass thiols to form *S*-nitrosothiols [4], but it is not certain whether this pathway is responsible for the formation *in vivo* of *S*-nitrosothiols. Proteins that are S-nitrosylated include glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [5,6], membrane receptors and several other enzymes and proteins [7,8].

GAPDH has received particular attention in view of the evidence that ·NO induces a linkage of NAD(H) to a cysteine residue in the active site [6,7]. This ·NO-dependent reaction has also been observed in a variety of different cellular extracts and intact cells [9], where it was not limited to the addition of exogenous ·NO but could be demonstrated after activation of endogenous NO-synthase [9-11].

The NAD(H)–cysteine linkage was suggested to be auto-ADP-ribosylation [12–15] or the covalent binding of the whole NAD(H) molecule [16]. Further studies showed that several ·NO-donating drugs induced the incorporation of radioactive nucleotide through a common mechanism possibly involving nitrosating agents (NO^+ or NO_2^+) [17].

Extending these observations, we observed that a free-radical generator 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH), which does not release ·NO or reactive nitrogen oxides, was indeed able to induce an NAD(H)-dependent modification of GAPDH. The present study was therefore undertaken to characterize a putative thiol intermediate responsible for the NAD(H) linkage to GAPDH exposed to radical attack.

MATERIALS AND METHODS

Materials

AAPH was obtained from Polyscience (Warrington, PA, U.S.A.). Rabbit muscle GAPDH (EC 1.2.2.12; crystalline suspension in ammonium sulphate) and NAD$^+$ were from Boehringer Mannheim GmbH (Mannheim, Germany). *N*-t-Butyl-α-phenylnitrone (PBN), catalase, L-cysteine, diethylenetriaminepenta-acetic acid, *N*-ethylmaleimide (NEM), mannitol, PMSF, thymidine and superoxide dismutase were obtained from Sigma Co. (St. Louis, MO, U.S.A.). [^{32}P]NAD$^+$ was obtained from du Pont de Nemours (Brussels, Belgium). [*carbonyl*-^{14}C]NAD$^+$ and [*adenine*-^{14}C]NAD$^+$ were obtained from Amersham (Bucks., U.K.). 3-Morpholinosydnonimine hydrochloride (SIN-1) was a

Abbreviations used: AAPH, 2,2′-azobis-(2-amidinopropane) hydrochloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NEM, *N*-ethylmaleimide; PBN, *N*-t-butyl-α-phenylnitrone; SIN-1, 3-morpholinosydnonimine hydrochloride; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine.

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gift from Cassella AG (Frankfurt, Germany). *S*-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP) was obtained from Alexis Corporation (Laufelfingen, Switzerland). Pure •NO gas was from SIO (Pomezia, Italy).

Preparation of apo-GAPDH

GAPDH was suspended (50–100 mg/ml) in 5 mM EDTA, pH 8.0. This preparation had $A_{280}:A_{260}$ ratios of 1.20–1.24, corresponding to 2.0–2.1 mol of NAD(H) per mol of the enzyme [18]. The removal of enzyme-bound NAD(H) was performed by activated charcoal chromatography. After purification, apo-GAPDH preparations had $A_{280}:A_{260}$ ratios of 1.9 ± 0.5, indicating that the enzyme was essentially NAD(H)-free [18]. The concentration of GAPDH was determined by the Lowry protein assay [19], taking the molecular mass of the monomer as 37000 Da. The thiol groups of apo-GAPDH were alkylated by incubation with 10 mM NEM at pH 8.0 for 15 min at 37 °C.

Red blood cell membrane preparation

Heparinized fresh human blood was obtained from healthy donors after informed consent. After centrifugation for 10 min at 1000 ***g*** and at 4 °C, plasma and buffy coat were removed and the red blood cells were washed three times with isotonic PBS, pH 7.4. The cells were then lysed in 10 vol. of ice-cold 5 mM sodium phosphate buffer, pH 8.0, containing 0.2 mM PMSF (to prevent proteolysis). Red blood cell membranes were washed three times with lysis buffer (48000 ***g*** for 10 min at 0 °C) and used for oxidative treatments.

Incubation with nitric oxide, SIN-1, peroxynitrite and AAPH

•NO solutions were prepared by bubbling 150 mM phosphate buffer, pH 7.2, with argon for 15 min and then with •NO gas for 5 min. In anaerobic experiments, the solution also contained GAPDH during the bubbling. In aerobic exposure, the •NO-saturated solution (approx. 1.9 mM at 25 °C) [20] was drawn with a syringe and injected into aerobic solutions containing erythrocyte ghosts or GAPDH.

SIN-1 is stable at pH 5.0, but readily releases •NO and superoxide at neutral to alkaline pH [20]. Ghosts and GAPDH were treated with SIN-1 in 10 mM phosphate buffer, pH 7.4.

Peroxynitrite was synthesized from sodium nitrite and H_2O_2 and stabilized by alkali as described by Radi et al. [21]. The peroxynitrite preparation was treated with manganese dioxide (30 min, 4 °C) to eliminate excess H_2O_2. The mixture was filtered three times to remove MnO_2. Peroxynitrite solution forms a yellow top layer by freeze fractionation, which was retained for further studies. The top layer typically contained 100–200 mM peroxynitrite. Peroxynitrite concentration was determined spectrophotometrically at 302 nm in 1.5 M NaOH ($\epsilon_{302} = 1670\ M^{-1} \cdot cm^{-1}$). Peroxynitrite solution was stored for 1–2 weeks in the freezer (−20 °C) with negligible changes in its concentration (less than 5%). The peroxynitrite anion ($ONOO^-$) is a relatively stable species but is a weak acid ($pK_a = 6.8$) and its protonated form (ONOOH) is unstable and rapidly decomposes at neutral to acidic pH ($t_{\frac{1}{2}} < 1$ s). For this reason peroxynitrite was added last and as a bolus to ghosts or to GAPDH solutions in 150 mM phosphate buffer, pH 7.2, and mixed immediately. Decomposed peroxynitrite was obtained by adding peroxynitrite to 150 mM phosphate buffer, pH 7.2, for 5 min at room temperature before the addition of ghosts or apo-GAPDH (reversed order of addition).

AAPH was added to ghosts and to GAPDH in 10 mM phosphate buffer, pH 7.4, and incubated at 37 °C to allow thermal decomposition.

To avoid contamination by transition metals, all buffers were treated extensively with Chelex 100 (Bio-Rad, Richmond, CA, U.S.A.) and contained 1 mM EDTA (final concentration) to avoid metal-catalysed nitrosating reactions [22]; replacement of EDTA with diethylenetriaminepenta-acetic acid did not affect the results.

Removal of oxygen for the anaerobic incubation was performed by bubbling the solutions with argon for 15 min.

[^{32}P]NAD$^+$ labelling of red blood cell membrane proteins

The incubation mixture for ^{32}P incorporation (80 μl final volume) contained 10 mM (or 150 mM for •NO gas and peroxynitrite) phosphate buffer, pH 7.4 (or pH 7.2 for •NO gas and peroxynitrite), 1 mM EDTA, 2 mM $MgCl_2$, 10 mM thymidine, 1 mM NADP, 1–2 μCi [^{32}P]NAD$^+$ (1 μM) and 20 μl of ghosts (about 80 μg of proteins). After incubation at 37 °C, the samples were centrifuged for 10 min at 48000 ***g*** and at 4 °C and washed twice in the same reaction buffer without radioactive NAD$^+$. To dissociate non-covalently bound NAD(H), membrane proteins (20 μl) were dissolved in 4 × loading buffer [23], heated in boiling water for 5 min and separated by SDS/PAGE [11% (w/v) gel]. Proteins on gels were stained with Coomassie Blue R-250. To reveal the ^{32}P-labelled proteins, the stained gels were dried and exposed to a standard Kodak X-ray film for about 18 h at −80 °C.

[^{14}C]NAD$^+$ labelling of purified apo-GAPDH

Purified apo-GAPDH (1 nmol) was incubated with a 5-fold molar excess of [*adenine*-^{14}C]NAD$^+$ (243 mCi/mmol) or [*carbonyl*-^{14}C]NAD$^+$ (53 mCi/mmol), with or without 100 μM SIN-1 or 50 mM AAPH. After incubation at 37 °C for 30 min the samples were denatured in SDS and subjected to SDS/PAGE as described above. Stained gels containing ^{14}C-labelled GAPDH were enhanced by treatment with 22% (w/v) 2,5-diphenyloxazole in DMSO and dried on Whatman 3MM paper. Excised regions of the gels containing the radioactive GAPDH were dissolved in 30% H_2O_2 and incubated at 60 °C overnight. After addition of scintillation liquid (5 ml of Opti-Fluor; Packard, Canberra Co., Meridien, CT, U.S.A.), the radioactivity was evaluated by using a Liquid Scintillation Analyzer (1600 TRI; Packard).

EPR spectroscopy

Spectra were measured on a Bruker ECS 106 EPR instrument. All samples contained 50 mM PBN added before the incubation with radical generators. Spectra were taken 2 min after the last addition. The samples were drawn into a gas-permeable Teflon tube with 0.81 mm internal diameter and 0.05 mm wall thickness (Zeuss Industrial Products, Raritan, NJ, U.S.A.). The Teflon tube was folded four times, inserted into a quartz tube fixed to the EPR cavity (4108 TMH) and exposed to air. Samples incubated anaerobically were drawn into a glass capillary tube and sealed at both ends. All measurements were performed at 37 °C. The intensity of the EPR signal was evaluated by double integration of the signals obtained after baseline correction by using the software supplied by Bruker (ESP 1600 data system).

RESULTS

Free-radical-induced ^{32}P labelling of red blood cell membrane proteins

Previous studies [12–17] have demonstrated that •NO-related oxidants produced a linkage of NAD(H) to a 37 kDa protein that was subsequently identified as the glycolytic enzyme

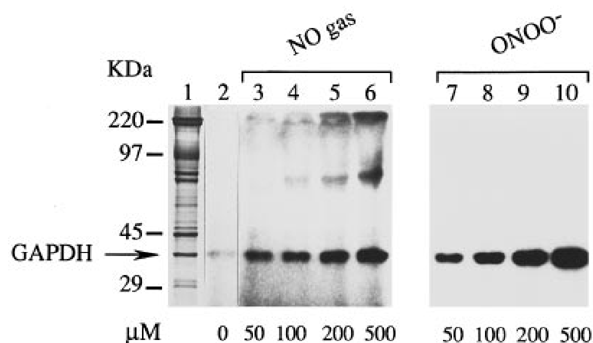

Figure 1 ^{32}P labelling of red blood cell membrane proteins induced by nitric oxide or peroxynitrite in the presence of [^{32}P]NAD$^+$

Lane 1, Coomassie Blue staining of red blood cell membranes analysed by SDS/PAGE; lanes 2–6, autoradiograms of ^{32}P-labelled proteins from ghosts treated with 0–500 μM $^{\bullet}$NO gas at 37 °C for 3 min; lanes 7–10, autoradiograms of ^{32}P-labelled proteins from ghosts treated with 50–500 μM peroxynitrite at 37 °C for 5 min. The positions of molecular mass standards are indicated in kDa (KDa) at the left. The position of GAPDH is indicated by the arrow.

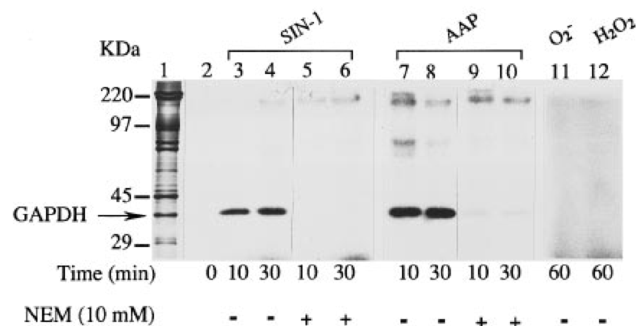

Figure 2 Time-dependent ^{32}P labelling of red blood cell membrane proteins induced by SIN-1 or AAPH in the presence of [^{32}P]NAD$^+$

Lane 1, Coomassie Blue staining of red blood cell membranes analysed by SDS/PAGE; lanes 2, 3, 4, 7 and 8, autoradiograms of ^{32}P-labelled membrane proteins treated at 37 °C with 50 μM SIN-1 for 0, 10 and 30 min (lanes 2, 3 and 4 respectively) or with 50 mM AAPH for 10 and 30 min (lanes 7 and 8 respectively); lanes 5, 6, 9 and 10, inhibition of ^{32}P incorporation into GAPDH by 10 mM NEM in samples treated with SIN-1 (lanes 5 and 6) or AAPH (lanes 9 and 10); lane 11, erythrocyte ghosts treated for 1 h at 37 °C with a superoxide generating system (1 mM xanthine/10 m-units/ml xanthine oxidase/40 μg/ml catalase; the production of superoxide was 7 μM/min); lane 12, ghosts treated with H_2O_2 (5 mM). The positions of molecular mass standards are indicated in kDa (KDa) at the left. The position of GAPDH is indicated by the arrow.

GAPDH. The effects of $^{\bullet}$NO gas and peroxynitrite were measured for the ^{32}P labelling of red blood cell membrane proteins incubated with [^{32}P]NAD$^+$. Figure 1 shows that increasing concentrations (0–500 μM) of $^{\bullet}$NO gas and peroxynitrite induced a dose-dependent increase in the labelling of a 37 kDa band. This protein was identified as GAPDH by immunoblotting analysis with anti-GAPDH antibodies [24]. High concentrations of $^{\bullet}$NO gas (500 μM) induced the formation of high-molecular-mass material due to the cross-linking of membrane proteins (Figure 1). Peroxynitrite at higher concentrations (1 mM or more) also induced the formation of cross-linked material (results not shown).

SIN-1 is a $^{\bullet}$NO-donor that at neutral to alkaline pH decomposes and generates nitric oxide and superoxide simultaneously [20]. These two radicals react very fast ($k = 6.7 \times 10^9$ $M^{-1} \cdot s^{-1}$) [25], resulting in the formation of peroxynitrite. As illustrated in Figure 2 (lanes 3 and 4), the treatment of red blood cell membranes with 50 μM SIN-1 modified the GAPDH inducing a time-dependent incorporation of ^{32}P.

To investigate the possibility that other free radicals, not correlated with $^{\bullet}$NO, could induce the same labelling of GAPDH, we treated red blood cell membranes with AAPH, an azo compound that generates carbon radicals at a constant rate by thermal decomposition [26]. In aerobic conditions, carbon radicals react with molecular oxygen to generate peroxyl/alkoxyl radicals [26,27]. As shown in Figure 2 (lanes 7 and 8), 50 mM AAPH induced a time-dependent incorporation of ^{32}P into the GAPDH of ghosts incubated with [^{32}P]NAD$^+$.

Other reactive oxygen species such as superoxide anion and H_2O_2 were unable to induce the incorporation of ^{32}P (Figure 2, lanes 11 and 12), thus excluding the participation of these species in AAPH- or $^{\bullet}$NO-dependent reactions.

To study the role of the cysteine residues of GAPDH, we performed the experiments previously described with SIN-1 and AAPH after alkylation of protein thiols with 10 mM NEM at pH 8.0 for 15 min at 37 °C). As shown in Figure 2, treatment of ghosts with NEM completely inhibited the incorporation of radioactivity induced not only by SIN-1 (lanes 5 and 6) but also by AAPH (lanes 9 and 10). This result was expected for the $^{\bullet}$NO-releasing compound, but proves that ^{32}P incorporation induced by AAPH is also cysteine-dependent.

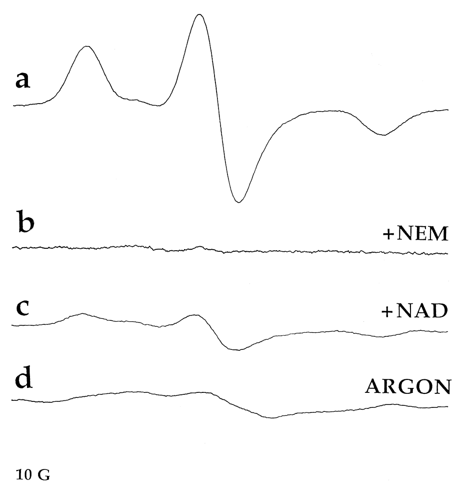

Figure 3 EPR spectra of radical adducts produced during the interaction of nitric oxide with GAPDH

(**a**) The sample was 1.5 mM $^{\bullet}$NO/apo-GAPDH (250 μM as monomer)/50 mM PBN/150 mM phosphate buffer/1 mM EDTA (pH 7.2); (**b**) sample as in (**a**), but in the presence of apo-GAPDH pretreated with NEM (10 mM, 15 min 37 °C); (**c**) sample as in (**a**), but with GAPDH reconstituted with 250 μM NAD$^+$; (**d**) sample as in (**a**), but incubated with $^{\bullet}$NO under anaerobic conditions as described in the Materials and methods section. Spectrometer conditions: frequency, 9.4 GHz; field modulation, 100 kHz; power, 20 mW; scan range, 100 G; time constant, 328 ms; gain, 3.2×10^5; modulation amplitude, 5 G; scan time, 84 s; number of scans, 15. In this and succeeding Figures, note that 1 G (gauss) $\equiv 10^{-4}$ T (tesla, the S.I. unit).

Spin trapping of GAPDH free radicals induced by $^{\bullet}$NO and $^{\bullet}$NO-related oxidants

The aerobic interaction of apo-GAPDH with authentic $^{\bullet}$NO gas, in the presence of the spin-trap PBN, led to the detection of the EPR spectrum shown in Figure 3(a). This spectrum is charac-

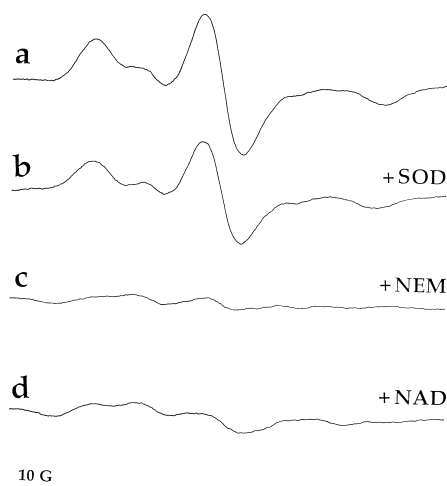

Figure 4 EPR spectra of radical adducts produced during the interaction of SIN-1 with GAPDH

(**a**) The sample was 100 mM SIN-1/250 μM apo-GAPDH/50 mM PBN/10 mM phosphate buffer/1 mM EDTA (pH 7.4); (**b**) sample as in (**a**), but in the presence of 500 units/ml superoxide dismutase (SOD); (**c**) sample as in (**a**), but in the presence of apo-GAPDH pretreated with NEM (10 mM, 15 min, 37 °C); (**d**) sample as in (**a**), but with GAPDH reconstituted with 250 μM NAD^+. Spectrometer conditions were as described in the legend to Figure 3.

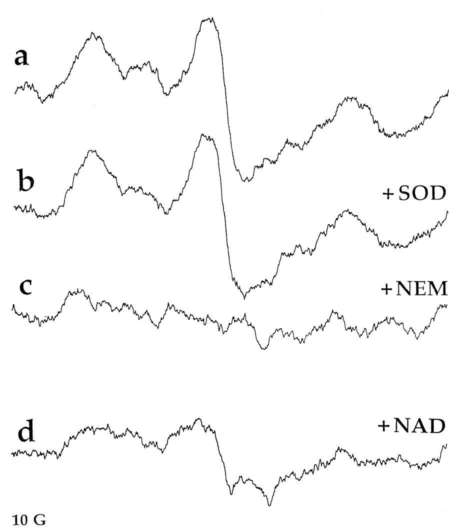

Figure 5 EPR spectra of radical adducts produced during the interaction of peroxynitrite with GAPDH

(**a**) The sample was 200 μM peroxynitrite/250 μM apo-GAPDH/50 mM PBN/150 mM phosphate buffer/1 mM EDTA (pH 7.2); (**b**) sample as in (**a**), but in the presence of 500 units/ml superoxide dismutase (SOD); (**c**) sample as in (**a**), but in the presence of apo-GAPDH pretreated with NEM (10 mM, 15 min, 37 °C); (**d**) sample as in (**a**), but with GAPDH reconstituted with 250 μM NAD^+. Spectrometer conditions were as described in the legend to Figure 3, except that the gain was 3×10^6.

teristic of a strongly immobilized nitroxide, suggesting that the trapped radical is constrained within the protein [28]. The anisotropic spectrum detected ($2a_{zz} = 68.6 \pm 0.5$ G; 1 G $= 10^{-4}$ T) was similar to the anisotropic spectra of PBN radical adducts of BSA ($2a_{zz} = 61$ G) [29–31], myosin ($2a_{zz} = 65$ G) and haemoglobin ($2a_{zz} = 61.6$ G) [32]. Interestingly, the previously described radical adduct of BSA was induced by reactions with ˙NO-related oxidants and was assigned to a protein thiyl radical [30,31]. To test whether the spectrum of Figure 3(a) could be due to the trapping of GAPDH thiyl radical(s), ˙NO gas was added to apo-GAPDH in which the cysteine residues had been alkylated by NEM. Under these conditions the formation of the PBN adduct was completely inhibited (Figure 3b), thus confirming the involvement of GAPDH thiyl radical(s).

Reconstitution of apo-GAPDH with NAD^+ decreased by 88 % the intensity of the PBN–GAPDH adduct induced by ˙NO gas (Figure 3c). One of the four cysteine residues per monomer of GAPDH (Cys-149) is especially reactive, is located in the catalytic site of the enzyme near to NAD(H) and is involved in enzyme catalysis [6,33]. Therefore the strong inhibition of the PBN–GAPDH adduct after reconstitution of the enzyme with NAD^+ suggests that ˙NO gas induced predominantly the formation of the Cys-149 thiyl radical.

The formation of the GAPDH thiyl radical by ˙NO gas required molecular oxygen, because apo-GAPDH incubated anaerobically did not induce the PBN–GAPDH adduct (Figure 3d).

The treatment of apo-GAPDH with SIN-1 leads to the trapping of a PBN radical adduct (Figure 4a) similar to that produced by ˙NO gas ($2a_{zz} = 67.8 \pm 0.6$ G). During ˙NO release by SIN-1, oxygen is consumed to form superoxide and these two radicals can interact further to form peroxynitrite. Under anaerobic conditions SIN-1 decomposition is inhibited [20] and we observed an 80 % decrease in the PBN–GAPDH adduct intensity if the reaction occurred anaerobically (results not shown).

We used authentic peroxynitrite to test whether this oxidant was able to induce the formation of GAPDH thiyl radical. As shown in Figure 5(a), peroxynitrite also induced a PBN–GAPDH adduct similar to that of ˙NO gas or SIN-1 ($2a_{zz} = 69.5 \pm 1.5$ G). The signal of the PBN–GAPDH adduct produced by peroxynitrite was, however, of approx. one-eighth the intensity.

The addition of superoxide dismutase decreased by 30 % the signal produced by SIN-1 (Figure 4b), but had no effect on the radical adduct produced by peroxynitrite (Figure 5b), thus supporting the hypothesis that peroxynitrite mediates at least in part the action of SIN-1. As previously observed with ˙NO gas, the PBN–GAPDH radical adduct produced by SIN-1 and by peroxynitrite was strongly inhibited in NEM-treated apo-GAPDH or after reconstitution of the apo-enzyme with NAD^+ (Figures 4c, 4d, 5c and 5d).

The PBN–protein radical adduct was not observed if decomposed 200 μM peroxynitrite was added to apo-GAPDH (the major by-products of peroxynitrite are nitrite and nitrate).

Substitution of 250 μM L-cysteine for GAPDH led, in the presence of 100 mM SIN-1, to the formation of a PBN–cysteine thiyl radical adduct ($a_N = 15.5$ G and $a_H = 3.5$ G), but the addition of 250 μM NAD^+ did not modify the signal intensity (results not shown). This control experiment demonstrates the specificity of the effect of NAD^+ on GAPDH, indicating the need for the active-site structure of GAPDH. These results agree with previous findings that the NAD^+–thiol linkage is not formed in the incubation of L-cysteine with the ˙NO-donor sodium nitroprusside [16].

In addition the ˙NO-donor SNAP, which releases large amounts of ˙NO on incubation with L-cysteine, led to the trapping of a PBN–GAPDH adduct with a hyperfine splitting constant similar to that produced by ˙NO gas, but the signal was of approximately one-eighth the intensity (signal intensity comparable to that of peroxynitrite). The spectrum was not observed without L-cysteine, under anaerobic conditions or in NEM-treated GAPDH (results not shown).

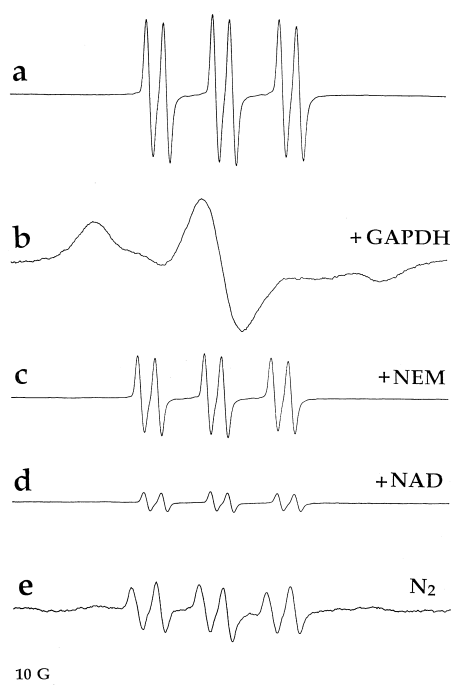

Figure 6 EPR spectra of radical adducts produced during the interaction of AAPH with GAPDH

(**a**) The sample was 100 mM AAPH/50 mM PBN/10 mM phosphate buffer/1 mM EDTA (pH 7.4); (**b**) sample as in (**a**), but in the presence of 350 μM apo-GAPDH; (**c**) sample as in (**b**), but in the presence of apo-GAPDH pretreated with NEM (10 mM, 15 min, 37 °C); (**d**) sample as in (**b**), but with GAPDH reconstituted with 350 μM NAD^+; (**e**) sample as in (**b**), but exposed to N_2. Spectrometer conditions were as described in the legend to Figure 3, except that the modulation amplitude in (**a**, **c**–**e**) was 1 G and the number of scans in (**a**–**c**) was 1.

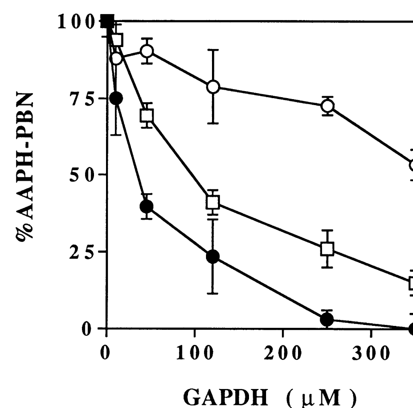

Figure 7 Scavenging effects of GAPDH on the adduct formed by AAPH and PBN

Symbols: ●, apo-GAPDH; ○, apo-GAPDH pretreated with NEM (10 mM, 15 min, 37 °C); □, apo-GAPDH reconstituted with equimolar NAD^+. The spectra were obtained after 2 min of incubation at 37 °C with 100 mM AAPH/50 mM PBN/10 mM phosphate buffer/1 mM EDTA (pH 7.4). Spectrometer conditions were as described in the legend to Figure 6(a). Points represent means ± S.D. ($n = 3$).

The reaction leading to GAPDH thiyl radical formation by ·NO-related oxidants was not dependent on H_2O_2, was not metal-catalysed and was not dependent on hydroxyl-like reactions, because it was not inhibited by catalase (500 units/ml), diethylenetriaminepenta-acetic acid (100 μM) or mannitol (100 mM).

Spin trapping of free radicals induced by AAPH

In the presence of PBN, AAPH produced the free-radical adduct shown in Figure 6(a). This adduct ($a_N = 15.50$ G and $a_H = 3.95$ G), obtained in air at 37 °C has been assigned to the alkoxyl radical of AAPH [27].

The addition of apo-GAPDH scavenged the AAPH radicals and decreased the PBN–AAPH adduct intensity in a dose-dependent manner (Figure 7). Interestingly, pre-treatment of the enzyme with NEM markedly decreased the protein scavenging ability. The concentration of apo-GAPDH necessary to produce a 50% decrease in PBN–AAPH adduct intensity increased 10-fold after alkylation of GAPDH cysteine residues (from 35 to 350 μM). Because NEM-alkylated GAPDH showed a residual scavenging ability, this residual activity was due to non-thiol residues of GAPDH.

Reconstitution of apo-GAPDH with NAD^+ decreased the scavenging ability of GAPDH (Figure 7), and the enzyme concentration producing a 50% decrease in the PBN–AAPH adduct increased approx. 3-fold in the holo-enzyme (from 35 to 94 μM). This effect cannot be ascribed to a direct interaction of NAD^+ with AAPH radicals because the interaction with AAPH radicals should increase and not inhibit the scavenging activity of GAPDH.

At the highest concentration of apo-GAPDH tested (350 μM) the PBN-alkoxyl adduct of AAPH was completely inhibited (Figure 7) and the radical trapped by PBN was a strongly immobilized nitroxide characteristic of protein adducts (Figure 6b). This PBN adduct ($2a_{zz} = 66.2 \pm 0.6$ G) was comparable to that produced by ·NO gas or by SIN-1 and might therefore be due to the thiyl radical adduct of GAPDH. According to this hypothesis, NEM-treated GAPDH not only showed a decreased scavenging ability for AAPH radicals (Figure 7), but also did not induce the formation of a PBN–GAPDH immobilized spectrum at high GAPDH concentrations. In this sample the only radical species trapped by PBN was the AAPH alkoxyl radical (Figure 6c). In addition, NAD^+-reconstituted GAPDH did not induce the formation of a PBN–GAPDH immobilized spectrum (Figure 6d), and PBN trapped the AAPH alkoxyl radical.

Anaerobic incubation of GAPDH with AAPH inhibited the formation of a PBN–GAPDH immobilized spectrum and PBN trapped a different free radical ($a_N = 15.6$ G and $a_H = 5.6$ G) assigned to the AAPH carbon radical (Figure 6e).

Analysis of the NAD(H) portion that modifies GAPDH treated with SIN-1 or AAPH

To characterize the free-radical-stimulated modification of GAPDH, we measured the radioactivity associated with the enzyme treated with NAD^+ labelled in the nicotinamide ([*carbonyl*-^{14}C]NAD^+) or in the adenine ([*adenine*-^{14}C]NAD^+). As shown in Table 1, a significant increase in the radioactivity associated with GAPDH after SDS/PAGE was observed after treatment with SIN-1 or AAPH, suggesting that both portions of the NAD^+ molecule can be linked to GAPDH. Moreover, the radioactivity associated with GAPDH was higher with adenine-labelled NAD^+ than with nicotinamide-labelled NAD^+. This preferential labelling was observed with both radical generators but was especially evident with AAPH (see the Ratio column in Table 1).

Table 1 Incorporation of radiolabel into GAPDH from [*adenine*-^{14}C]NAD^{+} or [*carbonyl*-^{14}C]NAD^{+}

Purified apo-GAPDH was incubated at 37 °C for 30 min with SIN-1 (100 μM) or AAPH (50 mM) in the presence of [*adenine*-^{14}C]NAD^{+} or [*carbonyl*-^{14}C]NAD^{+}. Values are the means ± S.D. for triplicate measurements.

	NAD bound (mmol/mol GAPDH)		
	[*adenine*-^{14}C]NAD^{+}	[*carbonyl*-^{14}C]NAD^{+}	Ratio (adenine/carbonyl)
Control	1.9 ± 1.7	2.0 ± 1.1	–
SIN-1	12.6 ± 1.0	8.5 ± 0.7	1.5 ± 0.2
AAPH	108.6 ± 9.0	36.0 ± 2.1	3.0 ± 0.5

DISCUSSION

The major finding of this study is that a linkage of NAD(H) to GAPDH can be induced not only by $^{\bullet}$NO-related species but also by AAPH, a free-radical generator that releases neither $^{\bullet}$NO or reactive nitrogen oxides. AAPH-induced NAD(H) linkage to GAPDH was observed in purified preparations of GAPDH as well as in erythrocyte ghosts, in which GAPDH is a membrane-associated protein, i.e. without the putative cytosolic NAD:cysteine transferase activity [34]. Therefore this study has focused on the automodification of GAPDH induced by NAD(H) in the presence of free radicals.

We performed spin-trapping studies with purified apo-GAPDH to characterize a putative thiol intermediate formed by $^{\bullet}$NO-related species and by AAPH. Treatment of apo-GAPDH with $^{\bullet}$NO gas, SNAP, SIN-1, peroxynitrite or AAPH induced the formation of a strongly immobilized nitroxide radical adduct of PBN inhibited by thiol alkylation (NEM) or by reconstitution of the apo-enzyme with NAD^{+}. The effects of NEM and NAD^{+} demonstrated that the PBN–GAPDH adduct was largely due to a thiyl radical arising from Cys-149.

Reactions leading to GAPDH thiyl radical formation

Aerobic incubation of GAPDH with $^{\bullet}$NO gas or $^{\bullet}$NO-donors induces a massive inactivation of the enzyme (more than 80–90 %) ([6,16]; C. Mallozzi, unpublished work). GAPDH inhibition is due primarily to the S-nitrosylation and, to a smaller extent (1–10 % from Table 1 and from [16]), to the NAD(H)-dependent modification of Cys-149. At neutral pH, $^{\bullet}$NO does not react with thiols to yield nitrosothiols [4] and is not the species directly involved in the incorporation of radiolabelled NAD^{+} into GAPDH [17]. Both these modifications require the $^{\bullet}$NO oxidation by molecular oxygen to higher nitrogen oxides ($2^{\bullet}NO + O_2 \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}NO \rightarrow N_2O_3$) or the use of nitrosating species (NO^{+}, NO_2^{+}, $ONOO^{-}$) [17]. In addition the PBN–GAPDH radical adduct was not observed if $^{\bullet}$NO gas was incubated anaerobically, suggesting that higher nitrogen oxides are probably involved in the oxidation of a GAPDH cysteine residue to a thiyl radical (several cysteine residues exist in GAPDH, but, under our conditions, only one of them, at the active site, is oxidized).

In a previous study [30] we presented evidence that the formation of a thiyl radical from S-nitrosylated serum albumin required a reductive activation by a low-molecular-mass thiol and molecular oxygen. Thiols were required for a reductive activation of the *S*-nitrosothiols and the release of $^{\bullet}$NO, whereas oxygen was probably necessary to form the higher nitrogen oxides responsible for the oxidation of thiols to thiyl radicals. In accordance with this mechanism, we observed that (1) GAPDH thiyl radical formation by $^{\bullet}$NO gas did not require the participation of free thiols, whereas the *S*-nitrosothiol SNAP induced the formation of the GAPDH thiyl radical only in the presence of L-cysteine, and (2) the GAPDH thiyl radical was formed by $^{\bullet}$NO gas or by SNAP only in the presence of oxygen.

The ability of peroxynitrite to oxidize protein thiols to thiyl radicals has been reported previously [31]. However, this oxidant in its anionic form is known to induce further oxidations of thiols to sulphinic or sulphonic acids [21]. These additional oxidative reactions can explain the relatively low yield of the PBN–GAPDH adduct observed with a bolus addition of peroxynitrite compared with the slow continuous production of $^{\bullet}$NO, superoxide and peroxynitrite by SIN-1.

GAPDH showed an NEM-inhibited and NAD^{+}-dependent ability to scavenge AAPH radicals, suggesting that Cys-149 in GAPDH is an important target of radicals generated by AAPH. The observation that the GAPDH thiyl radical was not trapped by anaerobic conditions suggests that AAPH peroxyl/alkoxyl radicals are more effective in thiyl radical formation than AAPH carbon radicals.

Reaction between the GAPDH thiyl radical and NAD(H)

There is some controversy about the mechanism of NAD(H)-dependent modification of GAPDH, it being either attributed to an auto-ADP-ribosylation mechanism [7,12–17,35,36] or resembling covalent binding of the whole NAD(H) molecule [16]. Our findings that AAPH and SIN-1 induced the incorporation of ^{14}C from NAD^{+} labelled either in the nicotinamide or in the adenine portion are consistent with the hypothesis [16] of a binding involving both portions of the NAD^{+} molecule. The mechanism of GAPDH thiyl radical attack to NAD(H) has not been thoroughly investigated but, as suggested by Stamler [7], reasonably oxidizable groups of NADH might be C-6 of the nicotinamide ring (the carbon *para* to the carbonyl group) or C-1′ of the ribose, i.e. the ribose carbon linked to nicotinamide. The former site of binding would produce a linkage of the entire NAD(H) molecule (and the ratio [*adenine*-^{14}C]NAD^{+}/[*carbonyl*-^{14}C]NAD^{+} should be unity), whereas the latter site of binding would produce the release of nicotinamide and the linkage of the ADP-ribose portion of the molecule, thus mimicking an ADP-ribosylation (ratio greater than unity). Our results suggest that Cys-149 of GAPDH can react with both these putative oxidizable groups of NADH but the binding at C-1′ of the ribose is slightly preferred. However, these conclusions are speculative and a detailed chemical analysis of the products released by NAD(H)-labelled GAPDH are necessary. By using sodium nitroprusside as $^{\bullet}$NO donor, McDonald and Moss [16] found that the products released from labelled GAPDH were NAD-like molecules not stable in solution and altered in the nicotinamide-ribose-phosphate portion.

In conclusion, from our results we suggest that the NAD(H)-dependent modification of GAPDH exposed to free radicals is mediated by a thiyl radical intermediate, which reacts with the neighbouring coenzyme to form cysteine–NAD(H) linkages. This mechanism implies that NAD(H) in the active site of GAPDH works as a trap for the thiyl radical and is consistent with the significant inhibition of the PBN–GAPDH adduct observed when apo-GAPDH was reconstituted with NAD^{+}.

Linkage of NAD(H) to GAPDH, if demonstrated *in vivo*, might mediate the cellular toxicity associated with elevated $^{\bullet}$NO production in activated macrophages [37] or the peroxynitrite-dependent neurotoxicity associated with *N*-methyl-D-aspartate receptor activation [38,39].

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