Characterization of the S-Denitrosation Activity of Protein Disulfide Isomerase*

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S-Nitrosoglutathione (GSNO) denitrosation activity of recombinant human protein disulfide isomerase (PDI) has been kinetically characterized by monitoring the loss of the S-NO absorbance, using a NO electrode, and with the aid of the fluorogenic NO probe, 2,3-diaminonaphthalene. The initial rates of denitrosation as a function of [GSNO] displayed hyperbolic behavior irrespective of the method used to monitor denitrosation. The $K_m$ values estimated for GSNO were $65 \pm 5 \mu M$ and $40 \pm 10 \mu M$ for the loss in the S-NO bond and NO production (NO electrode or 2,3-diaminonaphthalene), respectively. Hemoglobin assay provided additional evidence that the final product of PDI-dependent GSNO denitrosation was NO. A catalytic mechanism, involving a nitroxyldisulfide intermediate stabilized by imidazole (His169 $\alpha$-domain or His58$\alpha$-domain), which after undergoing a one-electron oxidation decomposes to yield NO plus dithiol radical, has been proposed. Evidence for the formation of thiol/dithiol radicals during PDI-catalyzed denitrosation was obtained with 4-((9-acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl. Evidence has also been obtained showing that in a NO- and O$_2$-rich environment, PDI can form N$_2$O$_3$ in its hydrophobic domains. This “NO-charged PDI” can perform intra- and intermolecular S-nitrosation reactions similar to that proposed for serum albumin. Interestingly, reduced PDI was able to denitrosate S-nitrosated PDI (PDI-SNO) resulting in the release of NO. PDI-SNO, once formed, is stable at room temperature in the absence of reducing agent over the period of 2 h. It has been established that PDI is continuously secreted from cells that are net producers of NO-like endothelial cells. The present demonstration that PDI can be S-nitrosated and that PDI-SNO can be denitrosated by PDI suggests that this enzyme could be intimately involved in the transport of intracellular NO equivalents to the cell surface as well as the previous demonstration of PDI in the transfer of S-nitrosothiol-bound NO to the cytosol.

Protein disulfide isomerase (PDI) was identified about 40 years ago (1). Although large levels of this enzyme are found in the endoplasmic reticulum, PDI is secreted from cells in which it associates electrostatically with the cell surface (2, 3). One of the most studied functions of PDI is its ability to catalyze isomerization and rearrangement of disulfide bonds in the endoplasmic reticulum, contributing to a proper folding of nascent proteins (4). Cell surface PDI was initially discovered in platelets (5), in which it plays a dual role in integrin-mediated adhesion and aggregation (6, 7), RSNO-mediated platelet inhibition, and GSNO denitrosation (8).

S-Nitrosothiols (RSNOs) are known nitric oxide (NO) donors. RSNOs range in size from low molecular weight, such as cysteine-NO and homocysteine-NO, to high molecular weight nitrosated proteins, such as serum albumin-NO. They are known to prolong NO half-life (9) and to act as a NO transport system in a cellular environment (10–12).

An additional novel activity of PDI is its ability to denitrosate RSNOs. On the cell surface, the RSNO denitrosation activity of PDI has been shown to play a role in the transfer of S-nitrosothiol-bound NO into cytosol (2, 13).

The present study was initiated because the RSNO denitrosation activity of PDI has not been well characterized. Here this was accomplished by directly monitoring the PDI-dependent loss in the S–NO bond via a NO electrode and the fluorogenic NO$_2$- and GS$_2$ probes. Recently it has been shown that PDI has copper binding activity (14). Our data suggest that PDI-bound copper does not play a role in the denitrosation activity of PDI.

We have also shown that the PDI stores the NO released from RSNOs, probably in the form of N$_2$O$_3$ (12) and can transfer it to intra- and intermolecular thiols. These significant findings could implicate PDI in both efflux and influx of RSNO-bound NO.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, reduced and oxidized glutathione, N-ethylmaleimide, diethylamine NONOate sodium salt, dithiothreitol (DTT), EDTA, diaminonaphthalene (DAN), neocuproine, sodium hydroxide, copper (II) sulfate, ammonium sulfamate, Sephadex G-25, and human hemoglobin were purchased from Sigma-Aldrich. 4-((9-Acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl (Ac-Tempo) was purchased from Molecular Probes (Eugene, OR). Bradford reagent was obtained from Bio-Rad.

Purification of Protein Disulfide Isomerase—Escherichia coli strain BL21(DE3) and expression vector pET-28a were used for expression of recombinant human PDI. The plasmid encodes a fusion protein containing the entire human PDI sequence with an N-terminal His$_6$ tag (15). Purification of recombinant PDI from the soluble fraction of cell lysate was done using Ni-CAM$^{TM}$ HC Resin (Sigma), a high capacity nickel affinity matrix. PDI bound to the resin was eluted using 250 mM imidazole in 50 mM Tris-HCl, pH 8.0, and was collected in 2-ml fractions. The fractions containing PDI were pooled and dialyzed Temp0, 4-((9-acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl; DTNB, 5,5'-dithiobis(nitrobenzoic acid); BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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The abbreviations used are: PDI, protein disulfide isomerase; PDI-SNO, S-nitrosated PDI; RSNO, S-nitrosothiols; GSNO, S-nitrosoglutathione; DTT, dithiothreitol; DAN, 2,3-diaminonaphthalene; NEM, N-ethylmaleimide; DEA-NO, diethylamine NONOate sodium salt; Ac-Tempo, 4-((9-acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl; DTNB, 5,5'-dithiobis(nitrobenzoic acid); BSA, bovine serum albumin; PBS, phosphate-buffered saline.
against 0.1 M potassium phosphate buffer, pH 7. Protein quantification was performed using the Bradford assay (16). The purity of protein was ascertained by gel electrophoresis and Western blot.

*PDI Assay Buffer*—PDI assay buffer contained 0.1 M potassium phosphate buffer, pH 7.0, and 2 mM EDTA. This buffer was used throughout the study unless otherwise specified.

**Synthesis of S-Nitrosothiol**—Prior to synthesis of S-nitrosothiol, [free thiol] in the GSH was determined with DTNB (17). A stoichiometric amount of oxidized NaNO₂ was reacted for 30 min. at 25 °C. Upon reaction completion, the pH of the solution was then adjusted to 7.4. Lastly, GSNO was recrystallized by the slow addition of ice-cold acetone and was resuspended in the appropriate buffer.

**Preparation of Oxyhemoglobin and Methemoglobin**—Oxyhemoglobin was prepared by reduction of human hemoglobin with dithionite in 100 mM potassium phosphate, pH 7.4, followed by chromatographic separation on Sephadex G-25 using the same buffer. Methemoglobin was prepared by oxidizing human hemoglobin with 5% excess of potassium ferricyanide in 100 mM potassium phosphate, pH 7.0, followed by chromatographic separation of unreacted species using Sephadex G-25 column equilibrated with the same buffer. Oxyhemoglobin and methemoglobin were prepared fresh prior to each experiment.

**Hemoglobin Assay**—The final product of GSNO cleavage by PDI was examined using the hemoglobin assay (18). Oxyhemoglobin assay is used to detect NO, which serves as an oxidizing agent thereby converting oxyhemoglobin to methemoglobin (19, 20). The reaction is accompanied by a decrease in absorbance at 542 and 580 nm and an increase at 630 nm, indicative of methemoglobin formation. The reaction mixture contained 30 μM oxyhemoglobin, 1.2 mM GSH, 100 μM GSNO (blank), and 2 μM PDI (sample), in 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA. NO generation was monitored by methemoglobin reduction to oxyhemoglobin and subsequent increase in 542 and 580 nm accompanied by a decrease in 630 nm. Assay solution contained 30 μM methemoglobin, 1.2 mM GSH, 100 μM GSNO (blank), and 2 μM PDI (sample), in 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. All spectra were recorded from 500 to 700 nm at specified time intervals. The extinction coefficient for oxyhemoglobin at pH 7.0 is 13,900 M⁻¹ cm⁻¹ and 14,400 M⁻¹ cm⁻¹ at 542 and 580 nm, respectively, whereas the GSNO extinction coefficient at 545 nm is 15 M⁻¹ cm⁻¹ (21) and is therefore considered insignificant. All measurements were performed using Agilent 8453 UV-visible spectrophotometer in a 1-cm path length quartz cuvette.

**Direct Monitoring of PDI Denitrosation Activity with UV-visible Spectroscopy**—The denitrosation activity of PDI was determined by monitoring the changes in absorption of GSNO (340 nm) as a function of time with different reducing agents. The reaction was performed in PDI assay buffer containing 100 μM GSNO and 3 μM PDI with varying concentrations of GSH (25 μM to 10 mM) to determine the ideal GSH concentration for optimizing GSNO denitrosation. The activity was also monitored with a fixed concentration of GSH (1.2 mM) and varying concentrations of GSNO (10–500 μM) to estimate its Kₘ. All measurements were performed using a Bio-tek Instruments ELx800, Ultra- microplate reader and Agilent 8453 UV-visible spectrophotometer at 25 °C.

**Detection of PDI Radicals with Ac-Tempo**—According to previous reports, Ac-Tempo, a paramagnetic nonfluorescent conjugate of nitroxide and acridine, interacts with glutathionyl radicals resulting in increased fluorescence of the acridine moiety of Ac-Tempo (22). Ac-Tempo concentration was determined by measuring absorbance at 359 nm (ε = 10.4 M⁻¹ cm⁻¹) using an Agilent 8453 UV-visible spectrophotometer. Fluorescence measurements were recorded on a Varian Cary Eclipse fluorescence spectrometer, with the excitation and emission wavelengths of 360 and 440 nm, respectively. PDI radical formation was detected by mixing stoichiometric amounts of PDI and GSNO (2 μM) with Ac-Tempo (10 μM) followed by a time-based measurement of radical generation. The possibility of Ac-Tempo reacting with GSNO coming from GSNO was eliminated by separate experiments that used DTT and GSH as reducing agents (data not shown). Also, additional evidence that PDI is responsible for GSNO denitrosation that results in PDI radical formation was conducted with the use of BSA (2 μM) instead of PDI. The standard plot of free radical formation was constructed by photolysis (355 nm) using an Applied Photophysics Laser Flash photolysis spectrometer; varying concentrations of GSNO (1–20 μM) were photolyzed for 40 s in the presence of Ac-Tempo, and the fluorescence was observed at 440 nm (excitation 360 nm). The standard plot was generated and used for thiol/dithiol radical quantification whenever required.

**Generation of a Standard Curve with DAN**—Varying concentrations of GSNO (200 nM to 25 μM) were incubated with DAN (200 μM) and HgCl₂ (100 μM) for 10 min in PBS (0.1 M, pH 7.4) at room temperature. The fluorescence readings were taken at 415 nm (λₑₓ, 375 nm). The quantification of released NOx when required, was estimated using this standard curve.

**Demonstration of the Ability of BSA and PDI to Store NO**—25 μM GSNO was incubated with 100 μM HgCl₂ for 10 min in the presence of 1, 2, and 4 μM BSA or PDI followed by the addition of ammonium sulfamate (0.1%) to remove the excess of HNO₂. DAN (200 μM) was then
added, and the fluorescence was monitored as a function of time at 415 nm (λex, 375 nm). The experiments were performed in 0.1 mM PBS buffer, pH 7.4, at 25 °C.

Preparation of PDI-reduced and S-Nitrosated PDI (PDI-SNO)—PDI was treated with 10-fold molar excess of DTT for 30 min at room temperature and dialyzed overnight in 20 mM phosphate buffer, pH 6.3, at 4 °C. The concentration of free thiols was measured with DTNB (17). PDI-NO was prepared by incubating reduced PDI with 5-fold molar excess of DEA-NO for 30 min at room temperature. The NO concentration was determined by NO meter using HgCl2 as described below.

Nitric Oxide Determinations Using NO Meter—Soluble nitric oxide was measured using the ISO-NO Mark II equipped with WPI MKII NO electrode. A standard curve for NO was generated by adding GSNO (10–100 μM) to 0.1 mM phosphate buffer, pH 7.0, containing 100 μM HgCl2.

PDI-NO (1.0 μM) was placed in the vial containing 0.1 mM phosphate buffer, pH 7.4, and the electrode was blanked using this mixture. At 20 s, 100 μM HgCl2 was added, and the NO generation was monitored until NO was no longer detected. In the parallel experiment, 2.0 μM PDI-reduced was added to PDI-NO to verify whether PDI alone could act upon PDI-NO. In a control experiment, BSA (2.0 μM) used instead of reduced PDI, did not result in NO generation (data not shown).

To demonstrate the involvement of the active-site thiols, NEM-blocked PDI was incubated with DEA-NO for 30 min at room temperature, generating NO-saturated PDI. 3.0 μM of this NO-saturated PDI was placed in the vial containing 0.1 mM phosphate buffer, pH 7.4, and the current was measured for 2 min. At 40 s, 100 μM HgCl2 was added to remove any S–NO present in the sample. Finally, at 130 s, 500 μM GSH was added to potentially scavenge N2O3 postulated to be present in the sample. At 400 s, 100 μM HgCl2 was added, and the NO generation was monitored.

Kinetic Characterization of PDI-catalyzed RSNO Denitrosation—The initial rates of denitrosation were monitored spectrophotometrically at 340 nm for the loss of S–NO absorbance with 3 μM human recombinant PDI and 1.2 mM GSH as a function of [GSNO] (10–500 μM). Initial rates versus [GSNO] data were well accommodated with the Michaelis-Menten equation and the apparent Km estimated for GSNO was 65 ± 5 μM (Fig. 1B). PDI (2 μM) denitrosation activity was also monitored by NO electrode by means of measuring NO released from GSNO (10–140 μM), and the apparent Km was estimated to be 40 ± 10 μM (Fig. 1C). The slightly higher Km observed spectrophotometrically is likely due to uncertainty in the initial denitrosation rates at low GSNO concentrations because of the low extinction coefficient of the S–NO bond at 340 nm (ε = 980 M−1 cm−1).

The end product of PDI-dependent GSNO denitrosation could be either NO− or NO2. To this end, hemoglobin assay was performed (18) for identifying the actual product of PDI denitrosation activity. Upon incubation of oxyhemoglobin with GSNO, GSH, and PDI, a time-dependent decrease at 542 and 580 nm and an increase at 630 nm were an indication of NO− production. The reaction was over in ~6 min. Inset, plot of decrease in absorbance at 540 nm as a function of time.

**FIG. 2. Monitoring NO− release by hemoglobin assay.** Oxyhemoglobin assay contained 30 μM oxyhemoglobin, 1.2 mM GSH, 100 μM GSNO, and 2 μM PDI in 100 mM potassium phosphate, 1.0 mM EDTA, pH 7.4. A decrease at 542 and 580 nm and a resulting increase at 630 nm were an indication of NO− production. The reaction was over in ~6 min. Inset, plot of decrease in absorbance at 540 nm as a function of time.

**RESULTS**

The Effect of GSH on PDI Denitrosation Activity—It is well established that free thiols like dithiothreitol (13, 24–27) or GSH (28) are required to maintain the thiol-disulfide exchange activity of PDI. The same is true for the PDI RSNO denitrosation activity because denitrosation was not observed in the absence of free thiols. In an attempt to determine the ideal RSNO/RSH ratio for maximal PDI denitrosation activity, the denitrosation of a constant amount of GSNO (100 μM) was monitored as a function of [GSH] in the presence and absence of PDI (Fig. 1A). The largest enzymatic rate/blank rate ratio (~6) was obtained with 1.2 mM GSH. This concentration of GSH was subsequently used in the Km estimations. Interestingly, no enzymatic denitrosation was observed when DTT or homocysteine was the reducing agent (data not shown).

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If NO− were produced, it would reduce methemoglobin that could be monitored by an increase in absorbance at 540 nm and 580 nm accompanied by a decrease at 630 nm. Because methemoglobin spectra in the presence of PDI and GSNO were stable for over 20 min, the amount of NO− possibly being produced was below the detection limit.

**PDI Radical Formation as a Result of GSNO Denitrosation**—To obtain additional evidence for the catalytic mechanism of PDI denitrosation activity, a nonfluorescent thyl radical probe, Ac-Tempo, was used. Previous studies have shown that Ac-Tempo, upon interacting with thyl radicals, produces a fluorescent signal. Here we set out to determine whether PDI would form a thyl radical in its active site as a result of GSNO denitrosation. First, GSNO was added to Ac-Tempo alone. The lack of fluorescent product formation (Fig. 3B, triangles) suggested that there is no spontaneous interaction between a probe and GSNO. However, when equimolar (2 μM) PDI plus GSNO were added to Ac-Tempo (10 μM), a continuous, time-dependent increase in fluorescence was observed. The rate of thyl radical formation (Fig. 3A, squares) closely paralleled NO production upon the mixing of equimolar (2 μM) PDI and GSNO, detected by the conversion of oxyhemoglobin to methemoglobin (Fig. 3A, diamonds). A comparison of the NO and thyl/dithyl stoichiometry indicated that 0.8 ± 0.12 mol NO produced per 3.0 ± 0.43 mol thyl/dithyl radical formed.

In control experiments, in which BSA was used instead of PDI, no thyl radical was detected (Fig. 3B, filled circles). Also, GSNO in the presence of a reducing agent GSH did not result in a change in signal upon PDI addition.

**PDI and BSA Act as a NO Sink**—Nudler and co-workers (12, 29) have previously demonstrated that hydrophobic domains of proteins like serum albumin can accumulate NO and O2 with which they react to form the potent S-nitrosating agent N2O3. Here we used DAN to test whether PDI could also perform a similar function. The amino group of DAN gets N-nitrosated by NO as well as by other oxides of nitrogen including NO2 and N2O3 (30, 31). The second amino group of DAN then reacts with nitrosated intermediate, yielding the highly fluorescent naph...
thotriazole derivative. Here 50 μM GSNO was incubated with HgCl₂ (32) to release NO in the presence of 1, 2, and 4 μM BSA (Fig. 4A) or PDI (Fig. 4B). With both PDI and BSA, the amount of naphthotriazole production, a direct indication of available soluble NOₓ, decreased with increasing [protein] (BSA or PDI).

Many studies indicate that NO and O₂ hydrophobic molecules concentrate in hydrophobic loci of biological milieus and react to produce the highly reactive nitrosating agent N₂O₃ (12, 29, 33, 34). Nudler and co-workers (12) have shown that the accumulated N₂O₃ in the hydrophobic pockets of BSA could nitrosate thiols. In the present case, the most likely candidate for S-nitrosation would be the active site thiols of PDI itself.

The next question we asked was whether PDI-SNO could be denitrosated by metals and another PDI. For these experiments, a NO electrode was used as a direct method for measuring soluble [NO] (35, 36).

**PDI Can Release NO from PDI-SNO**—In these experiments, a NO electrode was placed in the buffer containing NO-saturated PDI (1.0 μM) (Fig. 5, A and B), and the current was monitored for 20 s to ensure that there was not a spontaneous generation of NO occurring. At 20 s, HgCl₂ (100 μM) was added. This resulted in an instantaneous increase in current that corresponded to 4 ± 0.5 μM NO (Fig. 5A). A similar experiment was performed, using PDI (2 μM) instead of HgCl₂. This also resulted in an increase in current corresponding to 4 ± 0.5 μM NO (Fig. 5B). Therefore, the possible NO released from PDI-SNO was determined to be ~4.5 μM NO/~1.0 μM PDI, under our experimental conditions.
FIG. 5. Monitoring denitrosation rate using NO meter. A, PDI-SNO denitrosation by Hg. PDI-SNO (1 μM) was placed in the vial and the NO meter was blanked using this solution. After a stable signal was obtained, at 20 s, 100 μM HgCl₂ was added, and the NO generation was monitored until saturation was obtained. The blank that was used contained only buffer instead of PDI-SNO. B, PDI-SNO denitrosation by PDI was same as the above except that PDI was added instead of HgCl₂. C, PDI ability to store NO was tested by employing NEM-blocked PDI (3 μM) saturated with NO, as described under “Experimental Procedures.” At 50 s, addition of 100 μM HgCl₂ (thick line) or 500 μM GSH (dashed line) did not result in change in current. Addition of 500 μM GSH (thick line) or 100 μM HgCl₂ (dashed line) at 120 s resulted in a steady increase in current until saturation was reached. Inset, control obtained by addition of BSA instead of PDI to PDI-SNO, followed by the addition of HgCl₂.
To further investigate the possibility that PDI is able to store NO in the form of N$_2$O$_3$ in its hydrophobic pockets (12), we incubated NEM-blocked PDI with DEA-NO. This method ensured that there is no PDI-SNO formation. Upon addition of 100 μM HgCl$_2$ to NEM-blocked NO-saturated PDI, no change in current was observed. However, when 100 μM GSH was added to the sample, a slower increase of current was observed (Fig. 5C, dotted line), and the concentration of NO generated was found to be $\sim 10 \pm 1$ μM NO/1.0 μM PDI. The interpretation of these results is that in PDI that has its thiols blocked with NEM, intramolecular S–N cannot be formed. Therefore NO generated in the presence of HgCl$_2$ (Fig. 5C) is coming from GSNO that is formed from the reaction between N$_2$O$_3$ in NEM-PDI hydrophobic domains with added GSH. Addition of GSH in NEM-blocked PDI not exposed to DEA-NO (data not shown) did not produce any NO, supporting our interpretation. The same experiment was performed in the reverse order. Upon adding GSH to NEM-blocked PDI previously exposed to DEA-NO, no NO generation was observed (Fig. 5C). How-

The stability of PDI thiols was tested by first fully reducing it and then monitoring the thiol decay at room temperature by DTNB assay (Fig. 6A). It was observed that almost $\sim 50\%$ of the thiols were reoxidized in 2.5–3 h. Freshly purified PDI was also found to have 50–60% reduced thiols. When freshly purified PDI was nitrosated by DEA-NO and then separated by G-25 column, it showed a peak at $\sim 340$ nm (Fig. 6B, dark line). The peak disappeared after the addition of 100 μM Hg (Fig. 6B, dashed line). The corrected spectrum showed 50% PDI nitro-
sation (Fig. 6B, inset). To compare the activity of native PDI and PDI-SNO, the insulin turbidity method was employed. The assay was performed in the absence of any reducing agent. Whereas native PDI was found to be active for one turnover, no activity was observed with PDI-SNO (data not shown). PDI-
-SNO, once formed, was fairly stable for more than 2.5 h and could be denitrosated by DTT (data not shown).

**DISCUSSION**

The S-denitrosation activity of PDI, although demonstrated in several studies (2, 13), has not been well characterized. Here we set out to kinetically analyze the GSNO denitrosation ac-
tivity of human recombinant PDI. It has been well established that PDI requires thiol reductants to perform its thiol-disulfide exchange activity (37). Therefore, it is not surprising that the GSNO denitrosation activity probed here was also dependent on thiol-reducing agents. GSH turned out to be the best reduc-
ing agent when the loss in the S–NO bond absorbance at 343 nm was used to monitor the denitrosation. However, no significant denitrosation was observed when DTT or homocysteine was used as reducing agent (data not shown). This suggests that PDI denitrosation activity is specific to the presence of glutathione, which is the primary reducing agent in the cell with a concentration of $\geq 1$ mM (38). The PDI-dependent denitrosation reaction as a function of [GSNO] was probed via three independent methods: directly via the loss of the S–NO bond absorbance at 340 nm, directly by NO production via the NO electrode, or indirectly with DAN as we have reported earlier (39). The $K_m$ for GSNO estimated by spectrophotometric method was found to be $65 \pm 5$ μM (Fig. 1B), whereas the NO meter and DAN assays (39) gave a value of $40 \pm 10$ μM (Fig. 1C) and $65 \pm 5$ μM, respectively. Before proceeding further, we set out to determine the final product of GSNO denitrosation by PDI. Hemoglobin assay (Fig. 2) showed that it is NO$^-$ and not NO$^+$ being formed during the GSNO denitrosation by PDI, which is in agreement with a previous study presented on GSNO denitrosation (40). On the basis of results obtained, we propose the PDI denitrosation mechanism as shown in Scheme 1. In this mechanism, one of the vicinal thiols (Scheme 1, J) in the CXXC sequence of PDI active site undergoes a transnitro-
sation reaction with the RSNO substrate (Scheme 1, II). The second active site cysteine then attacks the nitrogen of the S–N=O yielding a nitroxyl disulfide intermediate (Scheme 1, III). The formation of this intermediate could be facilitated via delocalization of electrons in N–O to yield N=O, which could be stabilized via a positively charged side chain in the vicinity of the active site, such as His$^{160}$ in the $\alpha$-domain or His$^{588}$ in the $\alpha'$-domain (GenBank™ accession number AK027647). A one-electron oxidation results in the formation of nitroxyl disulfide radical (Scheme 1, IV), which could rearrange to yield an oxidi-
zized (–S–S–) PDI active site plus NO$^+$ (Scheme 1, V and VI). The formation of such a nitroxyl disulfide intermediate was proposed by Houk et al. in a recent computational study (41). In order for this mechanism to work, there is a requirement for an electron acceptor which could be the oxidized active site thiols of PDI dimer in the antiparallel arrangement as proposed by Solovyov and Gilbert (42). In support of this, it has been reported that PDI active site is the most oxidative ($\sim 180$ mV) (43) of all other members of thioredoxin-like family. In addition, it has been reported that oxidized active site thiols in DsbD...
matic turnover is an oxidized protein avidate and dithiyl radical in a formation of an unstable radical inter-
minate cysteine (II) at N of the S–N–O resulting in formation of a nitrooxyl disulfide intermediate (III), which could be stabilized by the nearby imidazole (ImH+) of His599. One-electron reduction by the second active site of PDI (subunit a’) leads to a formation of an unstable radical intermediate and dithiyl radical in subunit a’ (IV). The final product after one enzymatic turnover is an oxidized protein active site of subunit a and NO (V and VI) (40).

(PDI-like protein) can serve as electron transporter (44, 45). It was further proposed that flow of electrons within DsbD occurs via succession of disulfide exchange reactions (44). Additional evidence for thiyl radical production was obtained with the Ac-Tempo probe. When equimolar GSNO was mixed with PDI, thiyl/dithiyl radicals were generated (Fig. 3) supporting the proposed mechanism. In contrast, GSNO alone or BSA used instead of PDI did not result in production of radicals (Fig. 3B), nor did PDI and GSNO in the presence of GSH, which would reduce the thiyl/dithiyl radicals. In further support of the proposed mechanism, the rate of thiyl/dithiyl radical formation paralleled NO formation. The estimated stoichiometry of the reaction indicates that 3 mol of thiyl/dithiyl radical is formed per 1 mol of NO. This stoichiometry is well accommodated by Scheme 1, suggesting that the two active sites S’ plus the S–S’ (i.e. 3 mol of thiyl/dithiyl) plus 1 mol of NO (Scheme 1, IV and V) form in a concerted manner rather than sequentially.

The next question we asked was where does the NO released by PDI from GSNO go? The experimental evidence presented here suggests that the released NO accumulates in the PDI hydrophobic domains and is converted to N2O3. Nudler and co-workers (12, 29) have demonstrated that proteins like serum albumin can S-nitrosate proteins by acting as sinks for NO and O2 in their hydrophobic domains in which these molecules can efficiently react to produce N2O3, a potent nitrosating agent. Here we were able to show this using the NO2-specific reagent DAN. In this case, increasing amounts of both BSA (Fig. 4A) and PDI (Fig. 4B) were able to decrease the amount of NO released from GSNO by Hg2++. These results indicated that PDI, like BSA (12, 29), can act as a NO sink. These low yields could potentially be the result of such a tendency of PDI to get oxidized or of the PDI active site thios to be oxidized during protein manipulation. To verify this, the stability of reduced PDI was tested with DTNB assay. It was observed that almost half of the reduced PDI was reoxidized within 2 h (Fig. 6) after which it remained fairly stable. Furthermore, upon exposure of native PDI to DEA-NO, all of the free thiols of PDI could be nitrosated. Interestingly, when HgCl2 or PDI was added to this fully S-nitrosated PDI-SNO, ~50% of the S–NO was rapidly released as NO (Fig. 5, A and B, respectively).

We further confirmed that active site thios of PDI were essential for the release of NO from DEA-NO-exposed PDI. In these experiments, the free thiols of PDI were blocked with NEM. When PDI-NEM was exposed to DEA-NO and NO production was monitored in the presence of HgCl2, NO release was not observed. However, NO was released when thios in the form of GSH was introduced to this mixture (Fig. 5C, dotted line). Support for this observation was obtained by changing the order of GSH addition. When GSH was added first, there was no change in the signal. However, upon addition of HgCl2 rapid NO generation was observed, followed by time-dependent decay.

In conclusion, the results presented here have very important implications with respect to the role of PDI in the transport of NO equivalents from NO-producing cells to the serum and in the release of NO from RSNOs. Our results show that PDI denitrosates GSNO when NO levels are low, as is the case on the cell surface. However, in the presence of large amounts of generated NO, as in the intracellular environment of endothelial cells, PDI becomes a NO carrier via an N2O3-mediated auto-S-nitrosation of its active site thios. Because PDI is continuously excreted, this might be an important route for the transport of intracellular NO equivalents to the serum. Once excreted, PDI-SNO can be denitrosated by PDI yielding NO on the cell surfaces of endothelial cells and platelets. This could be a cyclic process because it has been speculated that excreted PDI may be recycled back into intracellular environment (3).

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