Peroxynitrite preferentially oxidizes the dithiol redox motifs of protein-disulfide isomerase

Received for publication, July 15, 2017, and in revised form, November 29, 2017. Published, Papers in Press, November 30, 2017. DOI 10.1074/jbc.M117.807016

Protein-disulfide isomerase (PDI) is a ubiquitous dithiol–disulfide oxidoreductase that performs an array of cellular functions, such as cellular signaling and responses to cell-damaging events. PDI can become dysfunctional by post-translational modifications, including those promoted by biological oxidants, and its dysfunction has been associated with several diseases in which oxidative stress plays a role. Because the kinetics and products of the reaction of these oxidants with PDI remain incompletely characterized, we investigated the reaction of PDI with the biological oxidant peroxynitrite. First, by determining the rate constant of the oxidation of PDI’s redox-active Cys residues (Cys53 and Cys397) by hydrogen peroxide (k = 17.3 ± 1.3 s⁻¹ at pH 7.4 and 25 °C), we established that the measured decay of the intrinsic PDI fluorescence is appropriate for kinetic studies. The reaction of these PDI residues with peroxynitrite was considerably faster (k = (6.9 ± 0.2) × 10⁴ M⁻¹ s⁻¹), and both Cys residues were kinetically indistinguishable. Limited proteolysis, kinetic simulations, and MS analyses confirmed that peroxynitrite preferentially oxidizes the redox-active Cys residues of PDI to the corresponding sulfenic acids, which reacted with the resolving thiols at the active sites to produce disulfides (i.e. Cys⁵³–Cys⁵⁶ and Cys³⁹⁷–Cys⁴⁰⁰). A fraction of peroxynitrite, however, decayed to radicals that hydroxylated and nitrated other active-site residues (Trp⁵², Trp⁶⁶, and Tyr³⁹³). Excess peroxynitrite promoted further PDI oxidation, nitration, inactivation, and covalent oligomerization. We conclude that these PDI modifications may contribute to the pathogenic mechanism of several diseases associated with dysfunctional PDI.

This work was supported in part by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Grants 2008/57721-3 and 2013/07937-8, Conselho Nacional de Desenvolvimento Científico Tecnológico (CNPq) Grant 573530/2008-4, and Pro-Reitoria de Pesquisa da Universidade de São Paulo (PRPUSP) Grant 2011.1.9352.1.8. The authors are members of Núcleo de Apoio à Pesquisa (NAP) Redoxima (PRPUSP) and the Research, Innovation and Dissemination Center (RIDC) Redoxima (FAPESP). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Figs. S1–S3 and Tables S1–S3.

1 Recipients of scholarships from Conselho Nacional de Desenvolvimento Científico Tecnológico.
2 Recipients of scholarships from Fundação de Amparo a Pesquisa do Estado de São Paulo.
3 To whom correspondence should be addressed: Dept. de Bioquímica, Inst. de Química, Universidade de São Paulo, Av Lineu Prestes, 748, São Paulo, CEP 05058-000, Brazil. Tel.: 55-11-3091-3873; Fax: 55-11-3091-3873; E-mail: oaugusto@iq.usp.br.

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1 Recipients of scholarships from Fundação de Amparo a Pesquisa do Estado de São Paulo, Brazil and 2Vascular Biology Laboratory, Heart Institute (InCor), School of Medicine, University of São Paulo, São Paulo, CEP 05403-000, Brazil.

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duction of disulfide bonds into nascent proteins, acting as a converging hub of several reactions involved in protein folding (4). In parallel, PDI oxidation impairs its reductase and to some extent isomerase activities (7) but increases chaperone activity (13).

However, the oxidation of PDI by low-molecular-weight biological oxidants has received limited attention in the literature. These oxidants are likely responsible for the oxidative post-translational modifications of PDI that have been detected under various conditions associated with oxidative stress, leading to the loss of PDI activity (14). For instance, PDI has been shown to be S-glutathionylated in cell cultures challenged with a nitric oxide donor (15), S-nitrosated during neurodegenerative diseases (16–18), adducted with 4-hydroxynonenal in the livers of rats treated with high-fat diet and ethanol (19) and in the lipid-rich areas of advanced human atherosclerotic lesions (20), carbonylated (21) and nitrated in aging mice (22), and nitrated in muscle biopsies of patients with mitochondrial diseases (23) and in the spinal cords of a mouse model of amyotrophic lateral sclerosis (24). These findings argue for a better understanding of PDI oxidation by biological oxidants with regard to the kinetics and products of these reactions.

The few available kinetic studies are limited to the oxidation of PDI dithiol by GSSG (12) and by hydrogen peroxide (25) and were not performed with full-length PDI but with mutated PDI domain a (W128F). This mutant, which contains only the Trp domain a (W128F). This mutant, which contains only the Trp main structure of PDI is essential for its high catalytic efficiency (20), we considered whether the decrease in PDI fluorescence can be used to follow the kinetics of the reaction of the full-length protein with biological oxidants. Hydrogen peroxide was used to test the methodology because the kinetics of its reaction with mutated PDI domain a has been studied previously (25). Next, the kinetics and products of the reaction between PDI and peroxynitrite5 were investigated because of its potential biological relevance.

Indeed, peroxynitrite is the potent oxidant produced by the diffusion-controlled reaction between nitric oxide and superoxide anion radical and is a major promoter of non-enzymatic protein thiol oxidation and protein nitration under physiological conditions (27, 28). In addition, peroxynitrite has been implicated in ER stress (20, 29). In other cellular compartments, PDI closely interacts with and regulates NADPH oxidase (4, 30), a superoxide radical anion producer, increasing the chances of peroxynitrite reacting with PDI. Here, we demonstrate the preferential oxidation of PDI redox-active Cys residues by peroxynitrite, which, after consecutive insults, causes further PDI oxidation, nitration, inactivation, and covalent oligomerization.

**Results**

**Kinetics of the reaction of PDI dithiols with hydrogen peroxide**

Taking into account the previous report showing that reduced PDI upon oxidation by excess GSSG shows a decrease in its intrinsic fluorescence accompanied by a blue shift (~4 nm) (13, 15), we treated reduced PDI (5 μM) with an excess of hydrogen peroxide (320 μM) and examined the changes in its fluorescence spectrum. As shown in Fig. 1A, the fluorescence of reduced PDI (black trace) decreased upon addition of 320 μM

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5 The term peroxynitrite refers to the sum of peroxynitrite anion (ONOO−; oxoperoxonitrate (−)) and peroxynitrous acid (ONOOH; hydrogen oxoperoxonitrate) unless otherwise specified.
**PDI oxidation by peroxynitrite**

hydrogen peroxide (red trace) but did not show a clear blue shift. Addition of DTT (500 \( \mu \text{M} \)) to the oxidized PDI recovered the initial PDI fluorescence (blue trace), confirming that PDI reactive thiols are the main targets of hydrogen peroxide (25).

Although the decay of the intrinsic fluorescence of PDI upon oxidation is small, it can be used for kinetic studies. Indeed, the decay of PDI fluorescence upon oxidation by 320 \( \mu \text{M} \) hydrogen peroxide with time fits to a single-exponential function and is reversed by DTT (Fig. 1B). Increasing the concentration of hydrogen peroxide (0.25–1.0 mM) increased the rate of fluorescence decay, but the overall fluorescence decrease and the recovery upon DTT addition were maintained (data not shown). Likewise, all of the measured fluorescence decays with time followed single-exponential functions from which the corresponding \( k_{\text{obs}} \) values were calculated. Plotting these values against hydrogen peroxide concentration provided a straight line (Fig. 1C), the slope of which permitted the determination of the second-order rate constant of the reaction between hydrogen peroxide and PDI reactive thiols as

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k = 17.3 \pm 1.3 \text{ M}^{-1} \text{s}^{-1}
\]

at pH 7.4 and 25 °C. No evidence for double-exponential decay was observed, indicating that both PDI reactive thiols (Cys\(^{53}\) and Cys\(^{397}\)) are oxidized by hydrogen peroxide with similar rate constants. Accordingly, the value of the second-order rate determined here for the full-length PDI (17.3 M\(^{-1}\) s\(^{-1}\)) was roughly twice that determined for the reaction between hydrogen peroxide and the mutated PDI a domain (9.2 M\(^{-1}\) s\(^{-1}\)) (25), which has only one redox-active site.

**Kinetics of the reaction of PDI dithiols with peroxynitrite**

After demonstrating the usefulness of the intrinsic PDI fluorescence for kinetic studies (Fig. 1), we applied it to investigate the reaction of reduced PDI (5 \( \mu \text{M} \)) with peroxynitrite. In view of the higher reactivity of peroxynitrite as compared with hydrogen peroxide (27, 31), we utilized a stopped-flow fluorometer and started with low peroxynitrite concentrations (2.5–10.0 \( \mu \text{M} \)). On the time scale of the experiment, PDI was not oxidized in the absence of peroxynitrite but in its presence was oxidized in a concentration-dependent manner as shown by the rate of decrease of PDI intrinsic fluorescence (Fig. 2A). The total fluorescence decay was also proportional to peroxynitrite concentration because it increased with oxidant concentration, varying from substoichiometric (2.5–7.5 \( \mu \text{M} \)) up to stoichiometric (10 \( \mu \text{M} \)) to PDI reactive thiols (5 \( \mu \text{M} \) PDI = 10 \( \mu \text{M} \) PDI reactive thiols). Relevantly, the total PDI fluorescence decay in the presence of peroxynitrite (10 \( \mu \text{M} \)) (\( \Delta F = -0.56 \text{ V} \)) was the same as that of excess hydrogen peroxide and twice that of 5 \( \mu \text{M} \) peroxynitrite (Fig. 2C). After PDI oxidation by substoichiometric and stoichiometric peroxynitrite, the initial PDI fluorescence was recovered by DTT addition as was the case of excess hydrogen peroxide (Fig. 2, B and C). In contrast, a higher peroxynitrite concentration, such as 20 \( \mu \text{M} \), caused a major increase in the overall decay of PDI fluorescence, which was only partially recovered upon DTT addition (Fig. 2, B and C).

These results suggested that PDI reactive Cys residues are the preferential targets of peroxynitrite in a process that is reversed by DTT up to the stoichiometric concentration of the oxidant and of PDI reactive thiols. Increasing the relative concentration of peroxynitrite over PDI reactive thiols results not only in their oxidation but also in PDI modifications that change its fluorescence spectrum in a manner not completely reversed by DTT.

To test the above suggestion, we performed experiments of limited proteolysis with trypsin under oxidizing and reducing conditions followed by electrophoretic analysis (Fig. 2E). Previous work has shown that upon oxidation of PDI reactive thiols the enzyme adopts a more open conformation, becoming more susceptible to proteolysis (13). Indeed, reduced PDI (10 \( \mu \text{M} \)) is fairly resistant to trypsin digestion (Fig. 2E, compare lanes 1 and 2). If reduced PDI is treated with a stoichiometric concentration of peroxynitrite relative to PDI reactive thiols (20 \( \mu \text{M} \)), the degree of digestion increases (Fig. 2E, compare lanes 5 and 6), and this increased susceptibility is almost completely reversed by the addition of DTT to the oxidized enzyme before trypsin addition (Fig. 2E, compare lanes 2 and 5–7). Peroxynitrite concentrations beyond 20 \( \mu \text{M} \) result in increased digestion and only partial recovery upon DTT addition as seen in the experiments performed with 200 \( \mu \text{M} \) peroxynitrite (Fig. 2E, lanes 11–13). In contrast, when reduced PDI is added to decomposed peroxynitrite (200 \( \mu \text{M} \)) (reverse addition), the digestion pattern resembles that of the reduced enzyme (Fig. 2E, lanes 3 and 4). Taken together, the above results (Fig. 2, A–C and E) confirm that the PDI reactive thiols are the preferential targets of peroxynitrite at substoichiometric and stoichiometric concentrations, but when in excess the oxidant also attacks other PDI residues. Accordingly, peroxynitrite has a complex reactivity and oxidizes biotargets by one- and two-electron mechanisms (see below) (27, 28, 32).

Therefore, to avoid using excess peroxynitrite over PDI reactive thiols, the second-order rate constant of the reaction between peroxynitrite and PDI reactive thiols was determined by the initial rate approach (33, 34). Such an approach could be used because the decay of PDI fluorescence was easily converted to the concentration of PDI whose reactive thiols were oxidized (Fig. 2, A–C). The initial rate values were calculated by many repetitions of the experiments shown in Fig. 2A, and the obtained values were plotted against peroxynitrite concentration, providing a straight line (Fig. 2D). The slope of the line divided by PDI concentration provided the apparent second-order rate constant of the reaction between PDI reactive thiols and peroxynitrite (\( k = (6.9 \pm 0.2) \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) at pH 7.4 and 25 °C). In all instances (for example, see Fig. 2A), the decay of PDI fluorescence followed a single-exponential function, indicating that both reactive Cys residues of PDI (Cys\(^{53}\) and Cys\(^{397}\)) react with similar rate constants, \( \sim 3.45 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) for each reactive thiol.

**Mechanistic aspects of the reaction of PDI with peroxynitrite**

To obtain further mechanistic insights on the reaction, additional experiments were performed. We first examined the reaction between peroxynitrite and native PDI, alkylated PDI, or oxidized PDI by following peroxynitrite decay at 310 nm to avoid changes in fluorescence due to PDI alkylation or oxidation. Because of the low molar absorptivity of peroxynitrite at this wavelength, relatively high concentrations of both peroxynitrite (30 \( \mu \text{M} \)) and PDI native, PDI alkylated, or PDI oxidized (30 \( \mu \text{M} \)) were used. As expected, peroxynitrite alone decayed at pH 7.4 (red trace) due to its acid-catalyzed decom-
position \((k = 0.35 \text{ s}^{-1})\) (35), but it was consumed faster in the presence of PDI (blue trace) (Fig. 3A). In contrast, PDI previously alkylated with iodoacetamide (black trace) did not affect the acid-catalyzed peroxynitrite decay (red trace), further indicating that PDI reactive thiols were the preferential targets of peroxynitrite. Previously oxidized PDI (dashed red trace) also did not alter peroxynitrite decay (Fig. 3A), excluding the possibility of the oxidation of PDI disulfide bond by excess peroxynitrite (36, 37) (see also below).

We also followed peroxynitrite decay at 310 nm in the presence of different concentrations of native PDI (Fig. 3B) to re-determine the second-order rate constant of the reaction by a different methodology. The initial rates of peroxynitrite decay were measured, and the values were plotted against PDI concentration (Fig. 3B). The slope of the line divided by peroxynitrite concentration provided the apparent second-order rate constant of the reaction between PDI reactive thiols and peroxynitrite \(k = (3.5 \pm 0.1) \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \) at pH 7.4 and 25 °C. This value is in good agreement with the value determined by the decay of intrinsic PDI fluorescence (Fig. 2D). Still, we consider the value determined by the intrinsic fluorescence of PDI more accurate because it was determined with lower concentration of reagents, minimizing the interference of secondary reactions.

Next, we determined the thiol content of PDI by treatment with an excess of 4,4′-dithiodipyridine (0.5 mM) (38) before and after reaction with different concentrations of peroxynitrite. Typically, reduced PDI contained 5.02 ± 0.2 thiols/protein, which is close to the expected 6.0 thiols/protein. The thiols of PDI (10 μM) were oxidized by peroxynitrite in a concentration-dependent manner as shown by plotting thiol depletion versus added peroxynitrite concentration (Fig. 3C). By fitting the

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**Figure 2. Peroxynitrite preferentially oxidizes the redox-active cysteines of PDI as revealed by PDI intrinsic fluorescence and limited proteolysis.** A, representative kinetics of reduced PDI (5 μM) oxidation by peroxynitrite (PN) (2.5–10 μM). B, representative kinetics of reduced PDI (5 μM) oxidation by 10 μM peroxynitrite (black curve) or 20 μM peroxynitrite (blue curve) and reduction of the oxidized samples by DTT (500 μM). To monitor the variation of the fluorescence upon oxidized PDI reduction, the samples were oxidized at the bench (15 min) and mixed with DTT in the stopped-flow instrument. C, variation of the intrinsic fluorescence of PDI upon oxidation by hydrogen peroxide (500 μM) or by peroxynitrite (5, 10, and 20 μM) and re-reduction of oxidized PDI by DTT. The experiments were performed as in B, determination of the second-order rate constant of the reaction of PDI redox-active thiol with peroxynitrite. Initial rates were determined by experiments similar to those shown in A. E, representative gel of limited proteolysis of PDI (10 μM) promoted by trypsin before or after treatment with the specified concentrations of peroxynitrite or DTT (500 μM). The lanes marked with R correspond to 200 μM decomposed peroxynitrite (reverse addition). All the incubations were performed in phosphate buffer (100 mM) containing DTPA (0.1 mM), final pH 7.4, at 25 °C. Analyses were performed as described under “Experimental procedures.” Error bars in C and D represent S.D. (n = 3 independent experiments).
results to two straight lines, it was observed that the steepest line had a slope of 1.1, a value that is not the one expected for the stoichiometry of the reaction between peroxynitrite and thiols (1.0:2.0) (Equations 1–4) (33, 39). These results were not surprising because the second-order rate constant determined for the reaction between peroxynitrite and PDI reactive thiols is not high enough to completely overcome peroxynitrite decomposition to hydroxyl and nitrogen dioxide radicals (Equation 3). This observation was confirmed by kinetic simulations using Gepasi v3.30 software (http://www.gepasi.org)6 and the equations shown in the text. To compare with the experimental values in C, all of the detectable PDI thiols were used in the plot of the simulation, although only the PDI reactive thiols are oxidized directly by peroxynitrite (Equation 2). All the incubations were performed in phosphate buffer (100 mM) containing DTPA (0.1 mM), final pH 7.4, at 25 °C. Analyses were performed as described under “Experimental procedures.” Error bars in B and C represent S.D. (n = 3 independent experiments).

Figure 3. Peroxynitrite preferentially oxidizes the redox-active cysteines of PDI but also decays to radicals. A, decay of 30 μM peroxynitrite monitored by its absorbance at 310 nm in the absence of PDI (red trace) and in the presence of 30 μM PDI (blue trace), the presence of 30 μM previously alkylated PDI (black trace), or the presence 30 μM previously oxidized PDI (dashed red trace). B, determination of the second-order rate constant of the reaction of PDI reactive thiols with peroxynitrite. Initial rates were determined by experiments similar to those shown in A (blue trace) with the specified concentrations of PDI. C, decrease of the total thiol content of PDI (10 μM) upon addition of the specified concentrations of peroxynitrite. D, kinetic simulation of the reaction between the reactive thiols of PDI (10 μM) with peroxynitrite (20 μM) using Gepasi software (http://www.gepasi.org)6 and the equations shown in the text. To compare with the experimental values in C, all of the detectable PDI thiols were used in the plot of the simulation, although only the PDI reactive thiols are oxidized directly by peroxynitrite (Equation 2). All the incubations were performed in phosphate buffer (100 mM) containing DTPA (0.1 mM), final pH 7.4, at 25 °C. Analyses were performed as described under “Experimental procedures.” Error bars in B and C represent S.D. (n = 3 independent experiments).

It should be noted that the first-order rate constant of Equation 4 is unknown, but it was considered to be rapid in the simulation (107 s–1) because PDI reactive thiols (Cys53 and Cys397) are located close to the resolving thiol residues (Cys56 and Cys400), respectively. It is also important to emphasize that in Equations 2 and 3 we represented only one of the redox-active dithiols of PDI for clarity, but the enzyme contains two (Cys53–Cys56 and Cys397–Cys400) (3), which apparently behave similarly with regard to oxidation (see also below).

Up to this point, the results indicated that peroxynitrite reacts preferentially with PDI reactive thiols (Cys53 and Cys397), producing their corresponding sulfenic acid derivatives that

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subsequently react with the resolving Cys residues of the redox-active domains (Cys\textsuperscript{56} and Cys\textsuperscript{400}, respectively) to form disulfides (i.e. Cys\textsuperscript{331–Cys\textsuperscript{56} and Cys\textsuperscript{397–Cys\textsuperscript{400}}). However, a fraction of peroxynitrite decomposes to produce hydroxyl and nitrogen dioxide radicals (Fig. 3D). The hydroxyl radical is the most potent biological oxidant and reacts with almost any organic molecule with second-order rate constants limited by the rate of diffusion. Nitrogen dioxide is a moderate oxidant but also reacts rapidly with several amino acids, particularly Cys, Trp, and Tyr (32, 41). Therefore, it is anticipated that even stoichiometric peroxynitrite as compared with PDI reactive thiols will preferentially oxidize PDI dithiols but will also promote nonspecific and irreversible PDI modifications.

To further support the above conclusion, we performed MS/MS analysis of the products of PDI oxidized by a stoichiometric concentration of peroxynitrite relative to PDI reactive thiols. PDI (10 \( \mu \)M) was treated or not with peroxynitrite (20 \( \mu \)M), and after 5 min of incubation, unreacted thiols were blocked with iodoacetamide (55 mM) in a denaturing medium. Following the removal of excess reagents, the samples were digested with trypsin and subjected to nano-ESI-Q-TOF-MS/MS analysis as described under “Experimental procedures” (42, 43). The tryptic digests of the untreated PDI samples presented mostly unmodified peptides except for oxidized Met residues that are expected to be oxidized during the LC-MS/MS workflow, hydroxylated Trp\textsubscript{128} and Trp\textsubscript{407}, and alkylated thiols (C\textsubscript{R}) in Cys\textsuperscript{312} and in the redox-active domains (Cys\textsuperscript{53}, Cys\textsuperscript{56}, Cys\textsuperscript{397}, and Cys\textsuperscript{400}). The latter peptides presented peaks at \( m/z \) values of 648.3051, 638.6209, and 511.2407, corresponding to a peptide \((386)KNVFVEYAPWCGHCK401\) with a monoisotopic mass of 1796.7804 and a charge of 3 (Fig. 4B), 2) a peak at \( m/z \) 599.9350 corresponding to a peptide \((387)NVFVEYAPWCGHCK401\) with a monoisotopic mass of 1796.7804 and a charge of 3 (Fig. 4B), and 3) a peak at \( m/z \) 482.2266 corresponding to the previous peptide with a missed cleavage site \((386)KNVFVEYAPWCGHCK401\) with a monoisotopic mass of 1924.8753 Da and charges of 3, 3, and 4, respectively (for instance, see Fig. 4A, Fig. S1, and Table S1).

The tryptic digests of PDI treated with stoichiometric peroxynitrite presented the same peptides containing the oxidized Met and hydroxylated Trp residues of the control. Additionally, three new intense peaks were present: 1) a peak at \( m/z \) 609.6186 corresponding to a peptide \((43)YLLVEFYAPWC\textsubscript{R}GHC\textsubscript{K}\textsuperscript{57} – 2H) with a monoisotopic mass of 1825.8320 Da and a charge of 3 (Fig. 4B), 2) a peak at \( m/z \) 599.9350 corresponding to a peptide \((387)NVFVEYAPWCGHCK401\) with a monoisotopic mass of 1796.7804 and a charge of 3 (Fig. S1), and 3) a peak at \( m/z \) 482.2266 corresponding to the previous peptide with a missed cleavage site \((386)KNVFVEYAPWCGHCK401\) with a monoisotopic mass of 1924.8753 Da and a charge of 4 (Table S2). Therefore, it was possible to identify peptides corresponding to both redox-active domains of PDI oxidized by stoichiometric peroxynitrite to the corresponding disulfides (Fig. 4B, Fig. S1, and Table S2).

Although disulfide formation was the major modification observed in PDI treated with stoichiometric peroxynitrite, approximately 2 orders of magnitude less intense spectra of peptides containing the disulfide with an additional modification were also clearly detected. Indeed, disulfide peptides with hydroxylated Trp residues (Trp\textsubscript{128} and Trp\textsubscript{407}) and with nitrated Tyr residues (Tyr\textsubscript{393}) were identified (Fig. S2 and Table S2). It is important to note that the tryptic digests of PDI oxidized by stoichiometric peroxynitrite also showed relatively intense spectra of both redox-active sites being alkylated. To estimate their relative yields in untreated and treated PDI, the experimental procedure and MS analysis were independently repeated three times. The MS intensity of each peptide of the active sites of each experiment was calculated based on the ratio between the extracted ion chromatogram of the peptide and the total ion chromatogram for each peptide. The intensity of each peptide was averaged, and the results are shown in Fig. 4C. Despite the high standard deviation values, there was a tendency of the alkylated peptides of PDI domains a and a’ to decrease 23 and 32%, respectively, in treated PDI as compared with untreated PDI. The average value (28%) is lower than the simulated PDI thiol consumption by peroxynitrite by a two-electron mechanism (57%) (Fig. 3D). Nevertheless, the values are compatible considering the complex workflow of MS experiments. The yields of the disulfide peptides were not compared because they are distinct peptides (Fig. 4B, Fig. S1, and Table S2) (44, 45).

Other possible PDI-thiol oxidation products, such as sulfenic, sulfinic, and sulfonic derivatives, were not detected under our experimental conditions. To exclude the possible presence of sulfenic derivatives, parallel experiments were run with the addition of dimedone (5,5-dimethyl-1,3-cyclohexadiene) (49, 50) immediately after PDI reaction with peroxynitrite followed by digestion and MS analysis. No dimedone adducts were detectable by inspection of the MS data (data not shown). Sulfinic and sulfonic derivatives were excluded by direct inspection of the MS data (Tables S2 and S3). The oxidation of PDI disulfides by stoichiometric peroxynitrite was also excluded by the experiments showing that oxidized PDI did not alter oxidant decay (Fig. 3A) (see also below).

Taken together, these results support our conclusion that stoichiometric peroxynitrite will preferentially oxidize PDI reactive thiols and promote nonspecific and irreversible PDI oxidation (Figs. 2 and 3). Indeed, PDI disulfides were the major oxidation products of PDI dithiols, and a small fraction of them also appeared hydroxylated (at Trp\textsubscript{52} and Trp\textsubscript{396}) and nitrated (at Tyr\textsubscript{393}) (Fig. 4, Figs. S1 and S2, and Tables S1 and S2) as predicted.

**Excess peroxynitrite-mediated PDI nitration, inactivation, and oligimerization**

We further investigated the modifications of PDI (10 \( \mu \)M) treated with excess peroxynitrite by SDS-PAGE and Western blotting (using an anti-nitrotyrosine antibody) (Fig. 5, A–C). The SDS-PAGE experiments showed that peroxynitrite promotes a concentration-dependent PDI oligomerization, proba-
bly through cross-link formation (42, 43, 51), that tends to reach a plateau at 100 μM peroxynitrite (Fig. 5, A and C). In parallel, PDI monomers and PDI oligomers were shown to be nitrated (Fig. 5B). As mentioned before, peroxynitrite-dependent nitration of non-metallic proteins, such as PDI, is mediated by the radicals that are produced from the proton-catalyzed decomposition of the oxidant (Equation 3) (27, 28, 32, 52). These radicals are expected to produce PDI-derived radicals; and in fact, spin trapping experiments with 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS) demonstrated the formation of PDI-derived radicals in PDI treated with an excess of peroxynitrite (Fig. 5D). In the absence of PDI, the EPR signal detected (top spectrum) was due to DBNBS oxidation by peroxynitrite (53). In the presence of PDI, the immobilized EPR signal detected (bottom spectrum) (ΔH = 0.33 (3H)) can be attributed to DBNBS/Tyr-PDI and/or DBNBS/Trp-PDI radical adducts because these adducts cannot be distinguished by the parameters of their EPR spectra (43, 51). Finally, we showed that PDI oxidation, nitration, and oligomerization by treatment with excess peroxynitrite leads to the inactivation of the insulin reductase activity of the enzyme (Fig. 5E). None of the PDI alterations promoted by peroxynitrite were detected in the presence of previously decomposed peroxynitrite (250 μM) (reverse addition) (Fig. 5).

Figure 4. Nano-ESI-Q-TOF MS/MS analysis of the alkylated and disulfide peptide 43YLLVEFYAPWCGHCK57 obtained from tryptic digests of reduced PDI(10 μM) untreated or treated with peroxynitrite (20 μM). A, MS/MS sequencing of the peak at m/z 648.3051, which corresponds to the alkylated peptide (monoisotopic mass, 1941.8906 Da) with a charge of 3, found in the tryptic digests of untreated PDI. R represents the carbamidomethyl group. B, MS/MS of the peak at m/z 609.6186, which corresponds to the oxidized peptide (monoisotopic mass, 1825.8320 Da) with a charge of 3, found in the tryptic digests of PDI treated with peroxynitrite. C, relative yields of carbamidomethyl and disulfide peptides of PDI untreated (control) or treated with 20 μM peroxynitrite (PN). The corresponding peptides of PDI domains a and a' are shown at the left and right sides, respectively. Error bars represent S.D. (n = 3 independent experiments). The incubations and analyses were performed as described under “Experimental procedures” and in the text. XIC, extracted ion chromatogram; TIC, total ion chromatogram.
To characterize the main stable products formed from PDI oxidation by excess peroxynitrite, PDI (10 μM) was treated with peroxynitrite (200 μM) and incubated for 5 min at 25 °C. Unreacted thiols were blocked with iodoacetamide (55 mM) in a denaturing medium. After removal of nitrate, nitrite, and excess reagents, the samples were digested with trypsin and subjected to nano-ESI-Q-TOF-MS/MS analysis as described under “Experimental procedures” (42, 43). As shown before (Fig. 4A and Table S1), tryptic peptides of the native monomer were essentially unmodified except for oxidized Met residues, hydroxylated Trp128 and Trp407 residues, and alkylated thiols. Excess peroxynitrite-treated PDI rendered tryptic peptides containing both redox-active cysteines oxidized to the disulfides and disulfide-containing peptides that were further oxidized and/or nitrated in addition to other modified peptides (Table S3). We also inspected the MS data for products that could be produced by oxidation of PDI disulfides by excess peroxynitrite, such as thiosulfonates and related compounds (36, 37), but they were not found (Table S3).

To determine whether excess peroxynitrite preferentially targets specific PDI residues, we estimated the yields of unmodified and modified peptides in untreated and treated PDI in three independent experiments (Tables S1 and S3 show one of the three experiments). The MS intensity of each unmodified and modified peptide contained in the tryptic digests from each type of experiment was calculated by the ratio between the extracted ion chromatogram of the peptide and total ion chromatogram for each peptide. Next, the intensities for each peptide were averaged, and the average intensity of each non-modified and modified peptide was plotted in Fig. 6A. It is clear that the alkylated peptides of untreated PDI practically disappeared, rendering the disulfides and modified disulfides of treated PDI (Fig. 6A). Next, the unmodified peptide of untreated PDI that decreased most (74%) to produce the corresponding nitrated peptide was the peptide containing Tyr63 (ALAPEYAK) (Fig. 6A). As shown before, the MS/MS spectrum of the corresponding nitrated peptide is shown in Fig. 6B. All of the modified peptides (Fig. 6A) rendered MS/MS acquisition that was comparable with that shown in Fig. 6B (data not shown). The other unmodified peptides that decreased considerably (33–22%) in treated PDI were those containing Tyr457, Tyr196, Tyr94, and Trp128, respectively (Fig. 6A). Interestingly, several of the more extensively modified residues were at or close to PDI active sites.

Discussion

PDI performs an extensive array of cellular functions, and its up-regulation under a variety of conditions has been considered to be a cellular defensive mechanism to restore proteostasis. Despite its up-regulation, PDI may become dysfunctional due to abnormal and/or excessive post-translational modifications caused by reactive oxygen- and nitrogen-derived species (14). However, the kinetics and the products of these reactions remain largely uncharacterized.
In this work, we showed that the decrease of the intrinsic fluorescence of PDI upon oxidation of its dithiols was useful to determine the second-order rate constants of their reaction with hydrogen peroxide (Fig. 1) and peroxynitrite (Fig. 2). In contrast to the behavior observed during PDI oxidation by GSSG (13, 15), the decrease in PDI fluorescence was not accompanied by a blue shift in either hydrogen peroxide- or peroxynitrite-promoted oxidation (for instance, see Fig. 1). This difference is likely due to the fact that glutathionylation of the redox-active thiols of PDI perturbs the environment of the tryptophan in the active sites (Trp52 and Trp396) to a greater extent than the disulfide bonds formed by hydrogen peroxide or peroxynitrite (Fig. 4 and Table S2). The crucial point for selecting the adequate kinetic approach in determining the second-order rate constants was establishing the conditions under which PDI dithiols were the major targets of the oxidant and could be reversed with DTT (Figs. 1 and 2). Based on these experiments, the pseudo-first-order approach could be applied

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**Figure 6.** Nano-ESI-Q-TOF MS/MS analysis and estimation of the modified peptides obtained in the tryptic digests of PDI (10 μM) treated with peroxynitrite (200 μM). A, relative yields of the unmodified peptides present in untreated PDI (control) and PDI treated with 200 μM peroxynitrite (PN). The unmodified and modified peptides are specified; R and D in subscript represent the carbamidomethyl and the disulfide group, respectively. B, MS/MS sequencing of the peak at m/z 454.2268, which corresponds to the peptide **38**ALAPEY(NO2)AK**65** (monoisotopic mass, 906.4447 Da) with a charge of 2. Error bars in A represent S.D. (n = 3 independent experiments). The incubations and analyses were performed as described under “Experimental procedures” and in the text. XIC, extracted ion chromatogram; TIC, total ion chromatogram.
to determine the second-order rate constant of the reaction of PDI dithiols with hydrogen peroxide \( (k = 17.3 \pm 1.3 \text{ M}^{-1} \text{s}^{-1}) \) at pH 7.4 and 25 °C (Fig. 1) but not with peroxynitrite. Even in small excess relative to PDI reactive thiols, peroxynitrite oxidized additional PDI residues, altering the intrinsic fluorescence of the protein (Fig. 2). Therefore, the second-order rate constant of PDI dithiol reaction with peroxynitrite was determined by the initial rate approach using low concentrations of the oxidant \( (k = (6.9 \pm 0.2) \times 10^4 \text{ M}^{-1} \text{s}^{-1}) \) at pH 7.4 and 25 °C (Fig. 2D). Our kinetic studies indicated that both redox-active cysteines (Cys53 and Cys397) are indistinguishable in reacting with hydrogen peroxide or peroxynitrite because the kinetics of the reactions follow single-exponential decays (Figs. 1 and 2). The second-order rate constants determined were expressed above in terms of PDI concentration, but if they are expressed in terms of PDI reactive thiol they become 8.65 and \( 3.45 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) for hydrogen peroxide and peroxynitrite, respectively. Thus, the results indicate that the decay of PDI fluorescence could be useful in determining the kinetics of the PDI dithiol reaction with other peroxides of biological significance, including lipid-, amino acid-, and other biomolecule-derived hydroperoxides (34, 54, 55).

Although both PDI redox-active Cys residues have a low \( pK_a \) (values reported are in the range of 4.4 – 6.7) (3), the second-order rate constants of each reacting with hydrogen peroxide \( (k = 8.65 \text{ M}^{-1} \text{s}^{-1}) \) or peroxynitrite \( (3.45 \times 10^4 \text{ M}^{-1} \text{s}^{-1}) \) are considerably lower than those determined for other protein-Cys residues of low \( pK_a \) such as those in peroxiredoxins \( (k \text{ in the range of } 10^5-10^6 \text{ M}^{-1} \text{s}^{-1}) \) (56–58). The results obtained with PDI are another example illustrating that the low \( pK_a \) of protein-Cys residues is not the only factor controlling their reactivity toward peroxides (31). Nonetheless, both PDI redox-active cysteines (Cys53 and Cys397) react \(~10\) times faster with both hydrogen peroxide and peroxynitrite than protein-Cys residues of proteins to which no major redox function has been attributed, such as bovine serum albumin \( (k = 1.1 \text{ and } 2.8 \times 10^3 \text{ M}^{-1} \text{s}^{-1}, \text{ respectively, at pH 7.4 and 37 °C}) \) (59). This fact and the high abundance of PDI in the ER and other cellular locations indicate that PDI dithiol oxidation by hydrogen peroxide (25) or by peroxynitrite may become physiologically significant.

Peroxynitrite-mediated PDI oxidation may become particularly relevant because it is not limited to the oxidation of PDI dithiols to disulfides, which is functional and reversed by biological reductants, but can also lead to PDI modifications that are resistant to cellular repair (Figs. 2, 5, and 6). We showed that although PDI reactive Cys residues are the main targets of peroxynitrite (Figs. 2–4) even concentrations of the oxidant equimolar to PDI reactive Cys residues led to additional modifications in close proximity to the disulfides (Fig. S2 and Table S2). These may be reduced by biological reductants, but the oxidized Trp residues \( (\text{Trp}^{52} \text{ and Trp}^{396}) \) and nitrated Tyr residue \( (\text{Tyr}^{393}) \) will not be repaired with unknown consequences. Apparently, peroxynitrite concentrations up to 40 \( \mu \text{M} \) did not promote a statistically significant inactivation of the reductase activity of PDI (Fig. 5E). It should be emphasized that the fraction of peroxynitrite that decomposes to radicals depends on the relative concentrations of the reactants. With 20 \( \mu \text{M} \) peroxynitrite and 10 \( \mu \text{M} \) PDI, the total yield of radicals estimated by kinetic simulation was 5.2 \( \mu \text{M} \) (2.6 \( \mu \text{M} \) each of hydroxyl and nitrogen dioxide radical) in good agreement with experimental values (Fig. 3, B and C). Simulating the reaction of 20 \( \mu \text{M} \) peroxynitrite with 500 \( \mu \text{M} \) PDI, it was found that 99.0% of the oxidant will react with PDI reactive dithiols, and 1.0% will decay to nitrate and radicals, producing a total yield of 0.12 \( \mu \text{M} \) radicals (Fig. S3). Because the fluxes of peroxynitrite under physiological conditions are expected to be low (27), PDI could act as a detoxifying route for peroxynitrite in environments rich in PDI, such as the ER. Nevertheless, it is anticipated that a small fraction of PDI will suffer oxidative modifications other than disulfide formation. Therefore, catalysis of repetitive cycles of peroxynitrite detoxification may result in PDI dysfunction.

Overall, the physiological implications of peroxynitrite-mediated PDI oxidation should be analyzed, bearing in mind the highly biased ratios of PDI toward reduction with \( in vivo \) estimates of a PDI disulfide/thiol ratio of 1:90 in the ER (7) and of a disulfide/thiol ratio of 0.3:100 in the cytosol (60). Thus, even minor increases in oxidized PDI could affect these ratios. The scenario, however, may differ among distinct cell compartments. In the ER, although the concentrations of PDI reach mM ranges, Ero1 and Nox4 mainly reduce molecular oxygen to hydrogen peroxide (3, 4). Thus, they do not constitute sources of superoxide anion radical that could react with nitric oxide to form peroxynitrite within the ER. However, peroxynitrite diffuses considerable distances in biological microenvironments (27, 61), eventually reaching the ER. Although low-level PDI oxidation in the ER might potentially favor PDI chaperone activity or substrate thiol oxidation to improve folding, PDI oxidation associates mainly with impairment of PDI isomerase activity (62). PDI may also translocate to the cytosol, although this is still controversial (63). In both ER and cytosol, PDI will face strong kinetic competition with abundant proteins, such as peroxiredoxins (31, 56–58), to react with peroxynitrite. Oxidized peroxiredoxins, however, can transfer their oxidizing equivalents to PDI (4), indirectly relaying peroxynitrite-oxidizing effects. Accumulated evidence also supports an extracellular location for PDI, relevant to thrombosis, platelet activation, and vascular remodeling (64, 65). Extracellular PDI is likely to be a minor fraction of total PDI pool (<2%) (66), whereas the known enzymatic sources of superoxide anion and nitric oxide may favor the encounter of peroxynitrite with extracellular PDI. Under these circumstances, the low levels of PDI and the expected higher flux of peroxynitrite are likely to result in PDI oxidation and even inactivation.

In fact, excess peroxynitrite over PDI reactive thiols promoted a concentration-dependent PDI nitrination, covalent oligomerization, and inactivation, probably through the intermediacy of PDI-derived radicals (Fig. 5) (27, 43, 51). The produced radicals were PDI-tyrosyl and/or PDI-tryptophanyl radicals as indicated by the EPR parameters of the DBNBS radical adduct spectra (Fig. 5D) (43, 51). Accordingly, MS and MS/MS analyses of the formed oxidation products from PDI (10 \( \mu \text{M} \)) treated with peroxynitrite (200 \( \mu \text{M} \)) showed that PDI was nitrated at several residues, particularly at Tyr43, Tyr457, Tyr196, Tyr94, and Trp128 (Fig. 6 and Table S3). PDI was also hydroxylated at Trp52 and Trp396 (Fig. 6 and Table S3). These results are consistent with the known reactivity of peroxynitrite (Equations 1–4) (Fig.
Peroxynitrite oxidizes thiols in a 1:2 stoichiometry (33, 67) and suffers a slower proton-catalyzed decomposition to hydroxyl and nitrogen dioxide radicals, which can oxidize, nitrate, and hydroxylate amino acid residues, particularly aromatic residues (27, 32, 41). It should be noted, however, that the side chains of all amino acids as well as the peptide bonds are targets of the extremely reactive hydroxyl radical (61, 68), but all of the other possible products were not searched for during the performed MS and MS/MS analyses. Also, we have not attempted to characterize the PDI products present in its covalent oligomers (Fig. 5, A–C) because their analysis by LC-MS/MS requires tools that are currently under development (69). Nevertheless, the characterization of these products will help in elucidating the chain of events leading up to PDI covalent oligomerization.

Intriguingly, most PDI residues found to be nitrated and/or hydroxylated by excess peroxynitrite are either at or near the redox-active sites (Figs. 6A and 7B and Table S3). This preference could suggest a possible interaction of peroxynitride with the PDI active sites. Such a possibility is difficult to reconcile with the fact that peroxynitride is a small and unstable molecule at physiological pH. More likely, the preference was dictated by...
the accessibility of the active-site residues to the solvent as indicated by a number of studies showing that when the oxidant is present in the bulk aqueous phase the solvent-exposed residues are more rapidly oxidized (for a review, see Ref. 68). It should also be emphasized that oxidation of PDI dithiols leads to a more open conformation of the enzyme (Fig. 2E) (13), probably facilitating the attack of peroxynitrite-derived radicals at the exposed active sites.

It is important to mention that the biologically ubiquitous CO₂ can compete with PDI for peroxynitrite. Indeed, CO₂ reacts relatively rapidly with the oxidant, producing carbonate radical and nitrogen dioxide in 35% yields (Equation 5) (27, 28, 32). Kinetic simulation of the reaction of 20 μM peroxynitrite with 500 μM PDI in the presence of the physiological concentration of CO₂ at pH 7.4 (1.3 mM) shows that 50.25% of the oxidant reacts with the redox-active motifs of PDI, and 49.25% reacts with CO₂, generating 6.90 μM radicals (3.45 μM each of carbonate radical anion and nitrogen dioxide radical) (Fig. S3). The carbonate radical (²E₀ = 1.8 V) is less oxidizing than the hydroxyl radical (²E₀ = 2.1 V) but is more specific and, together with nitrogen dioxide, tends to increase protein nitration (27, 28, 32, 52). Therefore, CO₂ is likely to decrease PDI thiol oxidation by two-electron mechanisms but increase PDI oxidation by one-electron oxidation mechanisms, eventually leading to PDI nitration, inactivation, and covalent oligomerization (Fig. S3). Such a possibility remains to be experimentally demonstrated

\[
\text{ONOO}^- + \text{CO}_2 \rightarrow 0.65 \text{NO}_3^- + 0.65 \text{CO}_2 + 0.35 \text{NO}_2^- + 0.35 \text{CO}_3^{2-}
\]

(Eq. 5)

Finally, it is important to note the relatively low intensity of the MS spectra of the tryptic peptides, both the alkylated and the disulfide peptides, that contain the dithiol in PDI domain a as compared with PDI domain a' (Figs. 4C and 6A, Fig. S1, and Tables S1–S3). We attributed these results to the intrinsic properties of the peptides of domain a, which make their detection by LC-MS/MS difficult (see "Results"). Similar difficulties were reported in other studies (46–48), some of which inferred differences in the behavior of PDI domains a and a'. This inference may be premature and should be confirmed by additional experimental support. In fact, further studies will be required to clarify the roles of individual PDI domains in the physiological functions of the enzyme (63). In this work, we used diverse methodologies that, taken together, indicate that PDI domains a and a' behave similarly with regard to peroxynitrite-mediated oxidation, and all of our data are consistent with the mechanism proposed in Fig. 7A.

In conclusion, our study showed that the decrease of the intrinsic fluorescence of PDI upon oxidation of its dithiols can be useful in determining their reactivity toward biological oxidants. By applying this property, we demonstrated that both redox-active Cys residues of PDI react with peroxynitrite at considerable rates, constituting an eventual detoxification route for this oxidant in PDI-rich environments, such as the ER. However, repetitive cycles of catalysis of peroxynitrite detoxification may result in the accumulation of PDI modifications that are not repairable and will likely disturb cell proteostasis.

In other environments or under conditions of high fluxes of peroxynitrite, the oxidant promotes further PDI oxidation, inactivation, and oligomerization. Because PDI has been implicated in both signaling (4, 30, 63, 65) and damaging events (14, 16, 17, 19, 21–24), its interaction with peroxynitrite may contribute to the pathogenic mechanism of several disease states.

**Experimental procedures**

**Materials**

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich, Merck, or Fisher and were analytical grade or better. The hydrogen peroxide solutions were prepared from stock immediately before use, and their concentrations were determined spectrophotometrically by reaction with horseradish peroxidase to produce compound I (Δε₉₀ = 5.4 × 10⁴ M⁻¹ cm⁻¹) (33). Trypsin Gold (mass spectrometry grade) was purchased from Promega (Madison, WI). Peroxynitrite was synthesized from sodium nitrite (0.6 M) and hydrogen peroxide (0.65 M) in a quenched-flow reactor as described previously (33). The peroxynitrite solution was treated with manganese dioxide to eliminate excess hydrogen peroxide. Peroxynitrite used in the experiments contained low levels of the contaminant nitrite (<30%) as determined by absorbance measurements (ε₃⁵⁵ = 24.6 M⁻¹ cm⁻¹). The concentration of the peroxynitrite was determined spectrophotometrically at 302 nm using an extinction coefficient of 1670 M⁻¹ cm⁻¹ (33). All solutions and buffers were prepared with Milli-Q water (Millipore) and treated with Chelex-100® resin (Sigma-Aldrich).

**Expression and purification of recombinant human PDI**

Histidine-tagged full-length human PDI1 was cloned into the pET28a vector (Novagen) and overexpressed in Escherichia coli strain BL21 (DE3) pLysS. PDI was first isolated and purified with an immobilized metal-affinity resin as described previously (51). The PDI-containing fractions were then pooled and further purified using a Q Sepharose column (70). The residue numbering used was for human PDI1 containing the ER retention sequence. All the experiments were conducted with freshly reduced PDI.

**PDI thiol reduction and alkylation**

Before use, PDI was reduced with 10 mM DTT for 1 h at 37 °C. The samples were then passed over a HiTrap desalting column (Amersham Bioscience) to remove excess DTT and other products. Protein elution was monitored by absorbance at 280 nm, and DTT elution was monitored by the increase in conductivity. Reduced PDI was concentrated by ultrafiltration (Amicon Ultra; cutoff, 30 kDa; Millipore). The concentrations of reduced PDI solutions were quantified at 280 nm using the ε value provided by the ProtParam tool (http://www.expasy.ch/tools/protparam.html) (ε₂₈₀ = 45,380 M⁻¹ cm⁻¹). For PDI alkylation, the protein was reduced with 10 mM DTT for 2 h at 4 °C; subsequently, iodoacetamide (55 mM) was added, and the reaction sample was incubated for 1.5 h at 37 °C.

**PDI thiol quantification**

Thiol quantification was performed spectrophotometrically after treatment of reduced PDI, alkylated PDI, or oxidized PDI
PDI oxidation by peroxynitrite

with an excess of 4,4′-dithiodipyridine (0.5 mM) (38) in the presence 1% SDS ($e_{325} = 21,400 \text{ M}^{-1} \text{ cm}^{-1}$). The thiol content from reduced PDI was typically 5.0 ± 0.2 thiols/protein, and thiol content decreased upon storage at −20°C. Therefore, freshly reduced PDI was used in all experiments.

Oxidized PDI

A few experiments were performed with oxidized PDI and peroxynitrite. To this end, PDI (50 μM) was previously oxidized with hydrogen peroxide (1 mM) in phosphate buffer, pH 7.4, containing DTPA (0.1 mM) at room temperature overnight (25). Excess hydrogen peroxide was removed by ultrafiltration (Amicon Ultra; cutoff, 10 kDa; Millipore). The concentration of oxidized PDI was determined as described above (the $e_{340}$ values for oxidized and reduced PDI are very similar). Thiol content of oxidized PDI was determined as described above; the value found was 0.5 thiol/PDI.

Kinetic studies of the reaction of PDI with hydrogen peroxide

Emission spectra (300 – 400 nm) of freshly reduced PDI (5 μM) in phosphate buffer (100 mM) containing DTPA (0.1 mM), pH 7.4 at 25°C, were recorded before and after the addition of various concentrations of hydrogen peroxide on a Hitachi F-2500 fluorometer (Hitachi High Technology America, Inc.) using an excitation wavelength of 295 nm. Following PDI oxidation by hydrogen peroxide, DTT (500 μM) was added to the mixtures before rescanning of the emission spectra to ascertain the reversibility of the processes. The kinetics of the reaction of PDI (5 μM) with various concentrations of hydrogen peroxide were performed under the same conditions and in the same instrument ($λ_{\text{excitation}} = 295 \text{ nm; } λ_{\text{emission}} = 340 \text{ nm}$). The $k_{\text{obs}}$ values were determined by fitting the traces to a single-exponential equation using OriginPro 8.0 software. At least three independent experiments for each substrate concentration were performed to calculate $k_{\text{obs}}$. The apparent second-order rate constant was determined from the slope of $k_{\text{obs}}$ values plotted against hydrogen peroxide concentrations using linear least-squares regression analysis.

Kinetic studies of the reaction of PDI with peroxynitrite

These studies were performed on an Applied Photophysics SX-18MV stopped-flow spectrometer (mixing time <3 ms) by following with time the intrinsic PDI fluorescence decay ($λ_{\text{excitation}} = 295 \text{ nm; } λ_{\text{emission}} > 320 \text{ nm}$) or by following peroxynitrite absorption decay at 310 nm ($e_{310} = 1600 \text{ M}^{-1} \text{ cm}^{-1}$) as previously described (39). Temperature was maintained at 25 ± 0.5°C, and the final pH of the spent reaction mixture was confirmed to be 7.4. It was necessary to determine the apparent second-order rate constant by the initial rate approach due to reasons that are discussed under “Results.” The initial rates were plotted against peroxynitrite or PDI concentrations to obtain the second-order rate constants. Kinetic simulations were performed with Gepasi v3.30 software (http://www.gepasi.org)6 (40).

Incubations for the analysis of PDI alterations caused by excess peroxynitrite

Unless otherwise stated, the reaction mixtures (100 μl) contained PDI (10 μM) in phosphate buffer (100 mM) containing DTPA (0.1 mM), pH 7.4, and peroxynitrite (10 – 250 μM). After 5 min at 25°C, aliquots of the spent reaction mixtures were analyzed as described below.

PDI inactivation

The inactivation of PDI was monitored by its reductase activity, which promotes the precipitation of the insulin B chain upon DTT reduction as described previously (51). In this procedure, PDI inactivation is monitored by the increase in the lag time required for insulin precipitation attaining an $A_{540 \text{ nm}}$ value equal to 0.1. The lag time required for insulin precipitation in the absence of PDI is taken as the control of complete PDI inactivation, and the turbidity increase due to insulin precipitation was spectrophotometrically monitored at 540 nm.

SDS-PAGE and Western blot analysis

For the limited proteolysis experiment, reduced PDI (10 μM) was incubated with peroxynitrite (20, 40, 200 μM) or 200 μM decomposed peroxynitrite (reverse addition). After 5-min incubation at room temperature, 0.02 μg of trypsin was added to the samples, which were further incubated for 40 min at room temperature. The digestion was terminated by the addition of phenylmethylsulfonyl fluoride (10 mM). In some samples, DTT (1 mM) was added before trypsin addition. Laemmli sample buffer was added to the spent reaction mixtures, and samples were heated at 95°C for 10 min and separated by 10% SDS-PAGE (13). The gels were stained overnight with Coomassie Blue and destained overnight with either distilled water or 1% acetic acid. For assays detecting PDI aggregation or nitration, Laemmli sample buffer was added to the spent reaction mixtures, and samples were heated at 95°C for 10 min and separated by 12% SDS-PAGE (51). Alternatively, the gels were transferred to nitrocellulose membrane and probed with an anti-nitrotyrosine antibody (Santa Cruz Biotechnology). Densitometry was performed using ImageJ v1.44p software (National Institutes of Health).

EPR experiments

The samples contained PDI (60 μM), DBNBS (10 mM), and DTPA (0.1 mM) in phosphate buffer (100 mM), pH 7.4. After addition of peroxynitrite (360 μM), the samples were incubated for 5 min at 25°C. Aliquots of the samples were transferred to flat cells, and the EPR spectra were scanned on a Bruker EMX instrument at 25 ± 2°C (51). The instrumental conditions used were: microwave power, 20 milliwatt; modulation amplitude, 2.5 G; time constant, 81.92 ms; conversion time, 81.92 ms; number of scans, 4; and scan rate, 1.2 G/s.

Nano-ESI-Q-TOF-MS-MS/MS analysis of trypsin hydrolysates of PDI treated with stoichiometric or excess peroxynitrite

PDI (10 μM) was incubated in the absence or presence of stoichiometric (20 μM) or excess (200 μM) peroxynitrite in phosphate buffer (100 mM) containing DTPA (0.1 mM), pH 7.4, for 5 min at 25°C. Immediately afterward, the sample (100 μl) was diluted with 900 μl of Tris-HCl buffer, pH 8.0, containing iodoacetamide (55 mM) and guanidinium chloride (6 M), and the samples were further incubated for 4 h at room temperature in the dark. After this time, excess iodoacetamide, guanidinium
chloride, and other low-molecular-weight compounds were removed by PD10 filtration. In all experiments, the protein fractions were dried in a speed vacuum, resuspended in ammonium bicarbonate buffer (50 mM), and digested with Trypsin Gold (protein/trypsin ratio of 50 μg:1 μg) for 12 h at 37 °C. Then a second aliquot of trypsin (protein/trypsin ration of 50 μg:1 μg) was added, and the samples were further incubated for 12 h at 37 °C. The hydrolysates were dried in a speed vacuum, redissolved in Milli-Q water containing 0.1% formic acid, desalted, and concentrated with a ZipTip C18 (Millipore) (43, 51). The salt-free hydrolysates were loaded onto an ACQUITY UPLC C18 (20 mm × 180 μm; 5 μm; Waters) nano-HPLC column coupled to a hybrid quadrupole-TOF-LC-MS/MS mass spectrometer (TOF-6600) with nanospray source (from AB Sciex instruments). The conditions for the nanospray source were: capillary voltage, 2.4 kV; dry heater; and 100 °C. The positive mode of the ionization source was used. Analyst TF software (v1.7.1) and PeakView software (v2.2) from AB Sciex were used for the analysis of the modifications. The mass tolerance in all experiments was ±10 ppm for MS analysis and ±0.05 Da for MS/MS analysis; the false discovery rate was ±1.0%.

Statistical analysis

All data are expressed as the mean ± S.D. of values determined in at least three independent experiments. Statistical significance was calculated using one-way analysis of variance and Tukey or Dunnett post-test using GraphPad 6.0 software (GraphPad Software, Inc.).

Author contributions—O. A. and F. R. M. L. conceived the study. A. S. P. and R. R. G. performed all of the reported experiments. A. I. performed the initial experiments establishing the conditions for the study. D. R. T. performed kinetic simulations. A. I. S. M. contributed to establish PDI expression and purification. All authors analyzed and discussed the results. A. S. P., F. R. M. L., and O. A. wrote the manuscript.

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