Crystal Structure of the Antioxidant Enzyme Glutathione Reductase Inactivated by Peroxynitrite*

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As part of our studies on the nitric oxide-related pathology of cerebral malaria, we show that the antioxidant enzyme glutathione reductase (GR) is inactivated by peroxynitrite, with GR from the malarial parasite Plasmodium falciparum being more sensitive than human GR. The crystal structure of modified human GR at 1.9-Å resolution provides the first picture of protein inactivation by peroxynitrite and reveals that this is due to the exclusive nitration of 2 Tyr residues (residues 106 and 114) at the glutathione disulfide-binding site. The selective nitration explains the impairment of binding the peptide substrate and thus the nearly 1000-fold decrease in catalytic efficiency (kcat/Km) of glutathione reductase observed at physiologic pH. By oxidizing the catalytic dithiol to a disulfide, peroxynitrite itself can act as a substrate of unmodified and bisnitrated P. falciparum glutathione reductase.

Nitric oxide (NO) is a pluripotent molecule that is involved in both cytoprotective and cytotoxic processes (1–4). In the latter case, the reaction of NO with metabolically generated reactive oxygen species exacerbates damage due to the formation of more potent agents such as peroxynitrite and nitrosoperoxycarbonate (5–7).

Peroxynitrite/peroxynitrous acid (ONOO−/ONOOH; pK a = 6.8) is produced by activated macrophages and other cells via the reaction of NO with superoxide (O2•−) at diffusion-controlled rates, and it easily crosses biological membranes (7–9). In vivo, peroxynitrite is estimated to have a half-life of <20 ms, primarily due to its reaction with CO2 and with target molecules, but also to the proton- or methionine-catalyzed isomerization to nitrate (10, 11). Nitrosoperoxycarbonate (ONOOCO2−), formed from ONOO− and CO2, has an even shorter half-life (6). Depending inter alia on the distance between the source of peroxynitrite and the target structure, the presence of CO2 can lead to potentiation or reduction of ONOO− reactivity (10, 12).

Peroxynitrite and its derivatives are powerful modifiers of proteins; the effects include nitrosation of cysteine; oxidation of cysteine, tryptophan, and methionine; and nitration of tyrosine (2, 4, 8). For an amazing variety of human diseases, high levels of nitrotyrosine are found in extra- or intracellular protein pools, which strongly implicates peroxynitrite as a pathophysiological agent (4). In this context, antioxidant enzymes (proteins that deal with the detoxification of the superoxide radical (O2•−) and its metabolic derivatives such as H2O2 and glutathione disulfide) have attracted special attention (13–18). With cerebral malaria, protein nitration in the brain is a typical post-mortem finding (19), which suggests that NO and its derivatives contribute to the pathogenesis and clinical outcome of the disease (3, 19–22).

For studying the NO- and peroxynitrite-related molecular pathology of cerebral malaria, we have chosen the antioxidant enzyme human glutathione reductase (hGR) and its counterpart from the malarial parasite Plasmodium falciparum (PfGR). The intracellular flavoenzyme hGR (EC 1.6.4.2) recycles oxidized glutathione (GSSG) to maintain high levels of reduced glutathione (GSH) by catalyzing the reaction GSSG + NADPH + H+ ⇄ 2 GSH + NADP+. Due to the fundamental role of GSH in scavenging and removal of deleterious reactive oxygen species, GR plays a crucial part in the antioxidant defense mechanisms of the cell (3, 22–24). GR is a possible target of NO carrier molecules by virtue of its inhibition in vitro by S-nitrosoglutathione (see Ref. 3), diglutathionyldinitrosoorb complex (see Ref. 3), and peroxynitrite (25, 26). Inhibition by S-nitrosoglutathione and diglutathionyldinitrosoorb complex was shown to occur via oxidation of the catalytically essential Cys154 to a sulfenic acid (R-SOH) and a sulfonic acid (R-SOOH), respectively (3). With regard to inhibition by peroxynitrite, hGR has not yet been studied. Bovine GR, however, was shown to be nitrated at 2 tyrosine residues/protein subunit, a modification that could be prevented by the substrate GSSG (26). It was hypothesized that the 2 residues were the equivalents of Tyr196 and Tyr114 in the structurally known hGR.

Here, we report the first picture of enzyme inactivation by peroxynitrite through a crystallographic analysis of modified...
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hGR at 1.9-Å resolution. Furthermore, we show that PGR (but not hGR) is an effective catalyst of ONOO- dependent oxidation of NADPH.

EXPERIMENTAL PROCEDURES

Materials—Peroxynitrite was synthesized from sodium nitrite and hydrogen peroxide using established protocols (27, 28) and was stored at –80 °C. Recombinant hGR, recombinant hGR with a Y114L mutation, and recombinant PGR were prepared as described (29). Enzyme kinetic studies were carried out at 25 °C in 47 mM potassium phosphate, 200 mM KCl, and 1 mM EDTA (pH 6.9) or in 100 mM potassium phosphate and 0.1 mM DPTA (pH 7.4).

Exposure of Cells and Enzymes to Peroxynitrite—Peroxynitrite was diluted with ice-cold 0.1% KOH to stock solutions ranging from 100 μM to 25 mM (Fo \( = 1.67 \times 10^{-3} \) cm⁻¹), and ONOO⁻ additions were made under continuous and vigorous stirring either by microbore injection or, for steady-state experiments, by infusion with a micropump at a rate of 1 ml/min (8). Because of protein instability, the samples could not be vortexed as recommended in Ref. 2. For modification reactions, GR solutions were at 2–20 units/ml (0.2–2 μM subunit) in 100 mM potassium phosphate and 0.1 mM DPTA (pH 7.4) at 25 °C. There were no detectable pH changes upon addition of peroxynitrite.

Absorption Spectroscopy—Flavin spectra of GR at 10–20 μM subunit concentration in 100 mM potassium phosphate and 0.1 mM DPTA (pH 7.4) were recorded at 25 °C. The ε values used for hGR (28) and PGR (30) were e645 = 11.5 and e640 = 0.3 mM⁻¹ cm⁻¹ for the oxidized enzyme (GR-S₁, e440 = 4.4 mM⁻¹ cm⁻¹ for the complex of reduced enzyme with NADPH (GR-(SH)₂-NADPH), and e425 = 4.2 mM⁻¹ cm⁻¹ for nitro-Tyr in GR. The latter value was pH-insensitive between pH 7.4 and 8.4 (see Ref. 26 and inset in Fig. 2).

Peroxynitrite as a Substrate—These assays were carried out at 25 °C in 100 mM potassium phosphate and 0.1 mM DPTA titrated with 2 M KOH to pH 8.5. The assay mixture contained 0.5 μM GR, varying initial concentrations of peroxynitrite (50–1000 μM), and 100 μM NADPH. Because the reaction product (probably nitrite) absorbs at 340 nm, NADPH oxidation could not be used to monitor reaction progress. Instead, the decrease in the peroxynitrite concentration was followed, or, for steady-state experiments, by infusion with a micropump at a rate of 1 ml/min (8). For enzyme inactivation, the samples could not be vortexed as recommended in Ref. 2. For modification reactions, GR solutions were at 2–20 units/ml (0.2–2 μM subunit) in 100 mM potassium phosphate and 0.1 mM DPTA (pH 7.4) at 25 °C. There were no detectable pH changes upon addition of peroxynitrite.

Inactivation of Glutathione Reductase by Tyrosine Nitration—The peroxynitrite inhibition of hGR and PGR (Fig. 1 and Table I) is qualitatively similar to that seen for bovine GR (26), with hGR exhibiting inactivation being proportional to nitrotyrosine formation (Figs. 1 and 2). However, as judged from their catalytic competence (kcat/Km), hGR and PGR are >300-fold more strongly inhibited than bovine GR at the same degree of nitration of 2 Tyr residues/subunit (Table I).

Structure determination revealed that both Tyr106 and Tyr114 are modified (see below). To distinguish their roles, we studied the previously characterized enzymatically active mutant Y114L (29). When exposed to peroxynitrite, the mutant lost <2% of its activity; and as judged from the absorbance change at 423 nm, <0.1 nitro group was incorporated per enzyme subunit. This unexpected result implies that the modification of Tyr106 depends on the presence of Tyr114 or, more likely, of nitro-Tyr114.

In the presence of 27 mM bicarbonate (which corresponds to 1.2 mM CO₂) at pH 7.4, the degree of inhibition by peroxynitrite was found to be 10-fold less both for hGR and PGR. This suggests that peroxynitrite itself and not nitrosoperoxycarbon-yl was the modifying species (6, 12). An alternative interpretation is that Tyr nitration slowed the reaction with NO₃⁻ decomposed by CO₂ (2, 6); this possibility will be studied using a stopped-flow apparatus for rapid mixing.

Peroxynitrite as a Substrate—In the oxidized form, GR is modified by an active-site disulfide (GR-S₁); and in the NADPH-reduced form, it is distinguished by an active-site dithiol (GR-(SH)₂ or GR-(SH)₂-NADPH complex). GR-(SH)₂-NADPH can

Detection of electron density maps with coefficients F(oxidized GR) = F(ox, GR) − F(ox, GR-ONOO⁻) = F(activated GR) and 2F(activated GR) − F(ox, GR-ONOO⁻) = F(activated GR) showed that the 2 active-site tyrosine residues, Tyr106 and Tyr114, were modified. There was no evidence for the modification of any other residue.

In the case of Tyr106, we found clear evidence for a nitro group at the ortho-position (3 or C-1) of the phenol group (see Fig. 3a). However, a more careful examination of the 2F, − F electron density map suggested a mixture of wild-type Tyr206 and nitratred Tyr106. This was further confirmed when preliminary positional and individual B-factor refinements with nitratred Tyr106 at full occupancy produced an average B-factor for Tyr206 of –30 Å² for peak atomic atoms and of –50 Å² for atoms of the 3-nitro group. To estimate the nitratred Tyr106/wild-type Tyr106 ratio, test refinements consisting of 30 cycles of conventional positional and 30 cycles of restrained B-factor refinements were carried out with nitratred Tyr106 at occupancies of 0.5, 0.6, 0.7, and 0.8, whereas the native conformation of Tyr106 was maintained as an alternate conformation at a complementary occupancy. The goal of this approach was (i) to find those combinations of occupancies that would produce near wild-type B-values for the wild-type conformation (–20 Å²), and uniform B-values for all atoms in all other occupancies and (ii) to minimize peaks in F, − F electron density maps. This approach led to the choice of 0.7 nitratred Tyr106 residues and 0.3 wild-type Tyr106 residues.

In the case of nitratred Tyr114, the 2F, − F electron density map was ill formed for substitutions. Inactive conformation and two other conformations with favorable torsion angles could be delineated. Furthermore, positive peaks in F, − F, electron density maps near the C-1 positions of the phenol rings of the two new conformations suggested that they represented 3-nitrotyrosine side chains. The occupancy of the side chain at position 114 was distributed among the three observed conformations (one for wild-type Tyr114 and two for nitratred Tyr114), and test refinements were carried out in a manner analogous to the procedure described for Tyr106. A model with occupancies of 0.2 for native Tyr114 and 0.4 for each of the nitrotyrosine conformers best met the above criteria and was accepted as the optimum.

Attempts to determine the structure of a complex of bis-nitro-GR with GSSG by soaking crystals in stabilizing solution of pH 8.0 containing either 10 or 20 mM GSSG for 48 h. Examination of F, − F, and 2F, − F electron density maps at 2.0 Å resolution yielded no indication for bound GSSG. We estimate that the limit of sensitivity of the difference Fourier is an occupancy of –20%.

RESULTS

Inactivation of Glutathione Reductase by Tyrosine Nitration—The peroxynitrite inhibition of hGR and PGR (Fig. 1 and Table I) is qualitatively similar to that seen for bovine GR (26), with NO₃⁻ inactivation being proportional to nitrotyrosine formation (Figs. 1 and 2). However, as judged from their catalytic competence (kcat/Km), hGR and PGR are >300-fold more strongly inhibited than bovine GR at the same degree of nitration of 2 Tyr residues/subunit (Table I).

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be clearly distinguished from GR-S₂ by its absorbance at 540 nm (Fig. 2). In the physiologic reaction, the dithiol of GR-(SH)₂ is reoxidized by dithiol-disulfide exchange with GSSG (29, 30, 39). Because peroxynitrite is known to oxidize thiols to disulfides (38), we studied whether peroxynitrite can replace GSSG as a substrate according to Equation 1.

\[
\text{NADPH} + H^+ + \text{ONOO}^- \rightarrow \text{NADP}^+ + \text{nitrile} + H_2O \quad \text{(Eq. 1)}
\]

As an initial test, GR-S₂ was incubated with NADPH in phosphate buffer (pH 7.4), which expectedly led to an absorbance band at 540 nm (Fig. 2). Upon addition of peroxynitrite, this absorbance disappeared, indicating that the active-site dithiol was reoxidized to the disulfide. The titration cycle (NADPH followed by peroxynitrite and NADPH again) could be repeated without a decrease in absorbance, indicating that the active-site dithiol was reoxidized to the disulfide. The presence of 100 μM NADPH had no influence on the rate and extent of tyrosine modification. 300 μM GSSG, however, protected the enzyme from nitration inactivation; only 0.3 NO₂ groups were incorporated per subunit.

### Table I

Kinetic characteristics of GRs before and after modification with peroxynitrite

| Enzyme species | Nitrotyrosine residues/subunit | \( k_{cat} \) for GSSG | \( K_m \) for GSSG | \( k_{cat}/K_m \) |
|----------------|-------------------------------|-----------------------|------------------|------------------|
| hGR            |                               | 165 ± 72              | \( 2.3 \times 10^6 \) s⁻¹ | \( 2 \times 10^6 \) M⁻¹ s⁻¹ |
| Modified hGR   |                               | 2.2 ± 5               | 1650 ± 95        | \( 3.0 \times 10^6 \) M⁻¹ |
| P/GR           |                               | 120 ± 5               | 95 ± 12          | \( 1.3 \times 10^6 \) M⁻¹ |
| Bovine GR      |                               | 2.1 ± 2               | 2310 ± 12        | \( 9.8 \times 10^5 \) M⁻¹ |
| Modified P/GR  |                               | 175 ± 4               | 42 ± 2           | \( 4.2 \times 10^6 \) M⁻¹ |
| bovine GR      |                               | 1.8 ± 1               | 109 ± 4          | \( 1.0 \times 10^6 \) M⁻¹ |

### Structure of hGR Modified by Peroxynitrite—Recombinant hGR inactivated by 75% with peroxynitrite (bistraturo-GR) was crystallized and structurally characterized at 1.9-Å resolution (Table II). The electron density maps revealed new density for Tyr₁⁰⁶ and Tyr₁¹⁴ in the GSSG-binding site of hGR and no unusual electron density features near any other residue. Specifically, there were no indications of dityrosine formation or sulfur oxidation. The selective nitration is consistent with the proposition that protein tyrosine nitration depends on the microenvironment of the targeted tyrosine residues (40). For both modified residues, we interpreted the observed electron density as a mixture of nitrated and wild-type tyrosines, and we were able to estimate the relative amount of each chemical species using systematic structural refinements that tested the plausibility of various models (see “Experimental Procedures”). Position 106 can be described well in terms of a ratio of 0.7:0.3 of nitrated Tyr₁⁰⁶ versus wild-type Tyr₁⁰⁶, with the overall conformation of nitrated Tyr₁⁰⁶ being very similar to that of the native conformation of Tyr₁⁰⁶ (Fig. 3, a and b). The nitro group points away from the core of the protein structure toward Arg₁⁰⁹, which provides for a favorable electrostatic interaction. Furthermore, nitrated Tyr₁⁰⁶ maintains its packing against Ala₁⁰⁹ and Met⁴⁰⁶ of the other subunit at the dimer interface, and there is no indication that the dimer interface is perturbed in this region or elsewhere.

In the case of position 114, we were able to delineate two distinct conformations of nitrated Tyr₁¹⁴ that differ from the native conformation of Tyr₁¹⁴, along with some residual unmodified Tyr₁¹⁴ (Fig. 3c). One of the two orientations of nitrated Tyr₁¹⁴ directs the nitro group into the bulk solvent region, whereas the other directs this group to the interior of the catalytic site, where it makes hydrogen bonds with active-site water molecules. A satisfactory model for this residue is a fractional ratio of 0.4:0.4:0.2 for nitrated Tyr₁¹⁴/wild-type Tyr₁¹⁴. Taken together, these crystallographic estimates add up to 1.5 nitrotyrosines, in good agreement with the 1.6 nitrotyrosines/hGR subunit derived from spectroscopic analysis (see “Experimental Procedures” and Fig. 2).
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**FIG. 2.** Absorption spectroscopy of bisnitrto-hGR. The dotted curve represents the oxidized form of peroxynitrite-treated hGR, and the solid curve represents the unmodified control (GR-SH). 423 nm is the wavelength used for the quantification of nitro-Tyr formation. Between 540 and 700 nm, the curve of the GR-(SH)2-NADPH complex is shown (---). In this wavelength range, the spectrum is indistinguishable for the unmodified and nitrated enzymes. The arrow indicates the cyclic appearance of absorbance at 540 nm upon addition of 200 μM peroxynitrite and its reappearance upon addition of 30 μM NADPH. The inset shows the difference spectra of bisnitrto-GR and the unmodified enzyme (---) and, for comparison, the spectrum of 3-nitrotyrosine at pH 8.4 (----).

**TABLE II**
Data collection and refinement for bisnitrto-GR

| Data collection                |                  |
|-------------------------------|------------------|
| Resolution limits (Å)         | 50 to 1.9        |
| Unique observations           | 41,212           |
| Multiplicity                  | 3.4              |
| Average I/σ                   | 10.6 (2.9)       |
| Rmerge (%)                    | 7.1 (35.3)       |
| Completeness (%)              | 96.1 (94.4)      |
| Refinement                    |                  |
| Resolution (Å)                | 20 to 1.9        |
| Reflections with F > 1σ       | 35,803           |
| Non-hydrogen protein atoms    | 3538             |
| FAD atoms                     | 53               |
| Water molecules               | 519              |
| Rcryst (%)                    | 16.6, 20.4       |
| r.m.s.d. bond lengths (Å)     | 0.005            |
| r.m.s.d. bond angles          | 1.20             |
| Overall anisotropic B-factor tensor (Å²) | B11 = 2.3, B22 = 5.2, B33 = -7.5, B13 = 1.4 |

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Impairment of Peptide Substrate Binding—The structural data suggest that the major functional impairment in bisnitrto-hGR is due to decreased affinity for GSSG as opposed to hindering an electron transfer step in catalysis. Evidence for this is that the Km of GSSG at pH 7.4 is >20-fold increased over that for the unmodified enzyme (Table I). In addition, soaks of bisnitrto-GR crystals in 20 mM GSSG failed to produce binding, whereas fixation of the substrate at high occupancy occurs in crystals of unmodified hGR (23, 34). The presence of nitrated forms of tyrosine residues can affect the productive binding of GSSG by imposing steric and, as emphasized here, electrostatic effects. Based on the pH of 7.0–8.0 reported for 3-nitrotyrosine residues in proteins (2, 41, 42), nitrated Tyr106 and nitrated Tyr114 will be largely in the negatively charged nitrophenolate form at the physiologic pH of 7.4. Charge repulsion between the modified enzyme and its peptide substrate would also explain the change in the Km of GSSG with increasing pH: the Km rises from 820 μM at pH 6.9 to >5000 μM at pH 8.0, whereas the Km for the unmodified enzyme is pH-independent in this range.

**DISCUSSION**

What Agent Is Responsible for Tyrosine Nitration?—Regarding the mechanism of hGR modification by peroxynitrite, our results imply that it involves a negatively charged or neutral nitrating species because (i) there are no negatively charged residues near the nitrated tyrosines; (ii) the GSSG site of hGR discriminates against positively charged ligands (43); and (iii) the rate of tyrosine nitration in GR is greater than for tyrosine peptides, which indicates a rate-promoting factor, such as local surface charge, in the protein (37, 40). Consequently, negatively charged nitrating species such as ONOO⁻ itself and its reactive adducts are plausible nitrating agents of hGR in vivo, as is the neutral nitrogen dioxide radical that results from the homolytic cleavage of peroxynitrite (44). Such a mechanism contrasts with the proposition that protein tyrosine nitration is caused by a positively charged nitronium (NO₂⁺)-like ion, as has been proposed for transition metal ion-catalyzed reactions of peroxynitrite (37, 40, 45, 46). It is possible, however, that Tyr106 is nitrated by a positively charged species if the nitration of Tyr114 occurs first, thus creating a local negative charge and changing the electrostatics of the active-site region.

Such introduction of a negative charge at the peroxynitrite-modified residue may represent a relevant mechanism also in the pathology of cell signaling (47, 48). In signaling cascades, irreversible nitration can affect the same tyrosine residues which are reversibly phosphorylated (45, 47–50). The two modifications have in common that the generated nascent negative charge has an impact on the conformation as well as on the binding behavior of the affected protein.

**Cystine Enzymes as Peroxynitrite Reductases**—P450GR represents an enzyme whose active-site dithiol catalyzes peroxynitrite reduction, probably to nitrite, with a kcat/Km ratio of >3 × 2 M. Scheiwein, unpublished data.
FIG. 3. Crystallographic evidence for the nitration of Tyr₁₀⁶ and Tyr₁₁⁴.

a, edge-on stereo view of nitrated Tyr₁₀⁶ (red) and wild-type Tyr₁₀⁶ (blue) in the final refined model of bisnitro-GR at 1.9-Å resolution superposed to a 1.9-Å resolution and 2Fᵦ – Fᵦ electron density contoured at 1.2σ. Residues of the other subunit are shown in green. b, stereo view of the face of nitrated Tyr₁₀⁶ (NIY 106), indicating the interaction of the nitro group with the guanidino group of Arg₁⁰⁹. c, stereo view of nitrated Tyr₁¹⁴/wild-type Tyr₁¹⁴ showing the three modeled conformations. The two conformations for nitrated Tyr₁¹⁴ have their nitrophenol groups flat in the plane of the paper (red), whereas the ring of Tyr₁¹⁴ in wild-type hGR is rotated ~60° from this plane (blue). Shown is a detail of the final refined model of bisnitro-GR at 1.9-Å resolution superposed to a 1.9-Å resolution and 2Fᵦ – Fᵦ electron density contoured at 1σ. d, location of Tyr₁⁰⁶ and Tyr₁₁⁴ in the GSSG-binding site of hGR (Ref. 31 and Protein Data Bank code 1GRA). GSSG (blue) and the 2 tyrosine residues (red) are shown along with other active-site residues such as the catalytic Cys₅₈ and Cys₆₃ (violet).
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$10^4$ M$^{-1}$ s$^{-1}$ (Equation 1). This enzyme activity reflects the chemical fact that peroxynitrite can oxide dithiols to disulfide, but not to higher oxidation states of sulfur (38). As a consequence, peroxynitrite reduction activity is probably an intrinsic property of all enzymes that oscillate between a dithiol and a disulfide form during the catalytic cycle. The list of such enzymes includes thioredoxin reductase and thioredoxins, lipoyl dehydrogenase, trypanothione reductase, as well as sulf-hydryl oxidase and asparaginase dehydrogenase (24). The comparison of $\mathcal{P}$GWR with the 12-fold less active hGR already indicates that the catalytic efficiency of peroxynitrite reduction might vary greatly among the listed cystine enzymes.

**Pathophysiological and Medicinal Implications—Peroxynitrite** is implicated in the pathophysiology of cerebral malaria (19–21) and other central nervous system disorders (16–18, 51–54). The effects of peroxynitrite as well as of peroxides are aggravated when the intracellular level of glutathione is decreased (54–56). In a rat model simulating early events in the pathogenesis of Parkinson’s disease, Barker et al. (25) observed that 60% depletion of GSH results in a decrease in GR activity. The peroxynitrite inhibition of GR in that report agrees with our results with the human enzyme. These findings are consistent with the notion that GSH is a scavenger of peroxynitrite. A lowered GSH level leads to increased inactivating nitration of GR. As a consequence, GSSG reduction is slowed down, and the competing reaction (GSSG excretion from the cell) establishes a vicious circle leading to lower GSH levels and lower GR activity (56).

The hypothesis that the nitration of hGR and $\mathcal{P}$GWR is involved in the pathogenesis of cerebral malaria can now be addressed by analyzing post-mortem tissues of cerebral malaria victims with respect to content and distribution of the modified enzyme species. By analogy to the studies on α-synuclein (57), a further step would be the analytical use of antibodies that specifically recognize the bisnitrated forms of host and parasite enzymes.

The findings reported here also suggest strategies for developing irreversible chimeric $\mathcal{P}$GWR inhibitors as lead compounds for new antimarial drugs (58). $\mathcal{P}$GWR is inhibited specifically by methylene blue, which apparently binds to the intersubunit cavity of the enzyme (30). As this drug-binding cavity is connected with the two active sites by intramolecular tunnels, work is in progress to construct a tripartite chimeric compound consisting of methylene blue, a chemical linker that snugly fits the chemistry of the tunnel, and a peroxynitrite donor (59). Methylene blue and the linker guarantee the specificity for the parasite enzyme target, and the peroxynitrite donor is expected to irreversibly modify the active sites.

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