How Covalent Heme to Protein Bonds Influence the Formation and Reactivity of Redox Intermediates of a Bacterial Peroxidase*

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Background: The impact of autocatalytically formed covalent heme to protein bonds on formation and reactivity of redox intermediates of a peroxidase was analyzed.

Results: Posttranslational modification significantly changes the reactivity of the ferric protein and of compounds I and II.

Conclusion: Upon covalent attachment, the capacity of compound I to oxidize one- and two-electron donors is enhanced.

Significance: First direct evidence about the relation between H2O2-triggered covalent heme to protein bond formation and enzyme reactivity.

The most striking feature of mammalian peroxidases, including myeloperoxidase and lactoperoxidase (LPO), is the existence of covalent bonds between the prosthetic group and the protein, which has a strong impact on their (electronic) structure and biophysical and chemical properties. Recently, a novel bacterial heme peroxidase with high structural and functional similarities to LPO was described. Being released from *Escherichia coli*, it contains mainly heme *b*, which can be autocatalytically modified and covalently bound to the protein by incubation with hydrogen peroxide. In the present study, we investigated the reactivity of these two forms in their ferric, compound I and compound II state in a multi-mixing stopped-flow study. Upon heme modification, the reactions between the ferric proteins with cyanide or H2O2 were accelerated. Moreover, apparent bimolecular rate constants of the reaction of compound I with iodide, thiocyanate, bromide, and tyrosine increased significantly and became similar to LPO. Kinetic data are discussed and compared with known structure-function relationships of the mammalian peroxidases LPO and myeloperoxidase.

The most striking feature of the mammalian peroxidases myeloperoxidase (MPO),3 eosinophil peroxidase (EPO), lactoperoxidase (LPO), and thyroid peroxidase is the existence of two covalent ester bonds between the prosthetic group and the protein in the functional, mature enzyme (1). Myeloperoxidase is unique in having an additional vinyl-sulfonium bond (see Fig. 1 (1, 2). These posttranslational modifications (PTMs) cause a distortion of the porphyrin ring from planarity, which is more pronounced in MPO (3–5) than in LPO (6). In MPO, the heme ring features a bow-shaped structure with less symmetry and a considerable out-of-plane location of the high-spin iron ion (3–5). Formation of these covalent heme to protein bonds has been proposed to occur autocatalytically (7–9) and has a deep impact on the biochemical and biophysical properties of these mammalian peroxidases (2, 10, 11). This includes significant modulation of the redox chemistry of these proteins, which provides the thermodynamic basis for the effective two-electron oxidation of halides, including bromide and chloride (11). The resulting reaction products, i.e. hypohalous acids, contribute to the antimicrobial role of MPO, EPO, and LPO in the innate immune system.

Analysis of the peroxidase-cyclooxygenase superfamily (12) suggested the occurrence of enzymatic domains with structural similarity to the vertebrate peroxidases (i.e. subfamily 1 including MPO, LPO, EPO, and thyroid peroxidase) in five of seven subfamilies. Interestingly, two new prokaryotic heme peroxidase subfamilies were found (12). Recently, the first bacterial representative of this subfamily was successfully produced in *E. coli* and biochemically characterized (13). The recombinant peroxidase from the cyanobacterium *Lyngbya* sp. PCC 8106 (LspPOX) was shown to have high similarities to mammalian LPO (see Fig. 1), including heme to protein linkages, redox and spectral properties as well as halogenation activity (13).

It was reported that the recombinant protein purified from *E. coli* was a mixture of two species with ~80% containing heme *b* (see Fig. 1B) and ~20% having the prosthetic group covalently bound to the protein via two ester linkages similar to LPO (Fig. 1D) (13). Most interestingly, this ratio shifted significantly when the recombinant protein was incubated with hydrogen peroxide. In this case, the majority (>70%) of the protein molecules showed covalently bound hemes. This strongly suggests autocatalytic formation of these covalent bonds triggered by hydrogen peroxide and resembles characteristics of mammalian peroxidases that also show intrinsic inhomogeneity regarding the heme to protein bonds. For example, the x-ray structure of human MPO purified from leukocytes clearly shows the side

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3 The abbreviations used are: MPO, myeloperoxidase; LspPOX, peroxidase from *Lyngbya* sp. PCC 8106; LspPOX (H2O2), peroxidase from *Lyngbya* sp. PCC 8106 incubated with hydrogen peroxide; LPO, lactoperoxidase; EPO, eosinophil peroxidase; X-, halide; PTM, posttranslational modification.
chain of Glu-242 (which is responsible for an ester bond linkage) in two distinct conformations (5). Moreover, both native and recombinantly produced LPO (7, 8) and EPO (9) contain heme b, which can be modified and covalently linked by external addition of 10–15 times molar excess of H₂O₂. A closer analysis of the effect of this posttranslational modification (PTM) in LPO, EPO, and MPO was so far hampered by the fact that the proteins could only be produced recombinantly in small amounts (or even failed in production) either in insect cell lines or animal cell factories. Moreover, the produced proteins often showed unsatisfactory heme occupancy. Now, for the first time, the cyanobacterial protein (LspPOX) with high similarity to LPO enables to directly monitor the impact of this PTM on the formation and reactivity of redox intermediates of a heme enzyme by a comprehensive multi-mixing stopped-flow study. LspPOX can easily and in relatively high amounts be produced in E. coli and has almost 100% heme occupancy (13). Moreover, upon incubation with hydrogen

**FIGURE 1. Structures of active sites and prosthetic groups of HRP, LPO, and MPO.** The structure of active site of HRP (A) and (unmodified) heme b (B) is shown. The structure of the active site of bovine LPO (C) and its modified prosthetic group (ester linkages between Asp-109 and the carbon of the 5-methyl group of pyrrole ring C as well as between Glu-258 and the carbon of the methyl group of pyrrole ring A) (D) are shown. Shown are active site structure of human MPO (E) and its modified prosthetic group (two ester linkages similar to that in LPO and an additional sulfonium ion linkage between the sulfur atom of Met-243 and the β-carbon of the vinyl group of pyrrole ring A) (F). Figures were constructed using the coordinates deposited in the Protein Data Bank codes: 1ATJ (HRP), 2GJ1 (LPO), and 3F9P (MPO). Please note that covalent heme to protein bonds in LspPOX (H₂O₂) correspond to that in LPO (D).
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Hydrogen peroxide obtained as 30% solution was purchased from Sigma-Aldrich. Hydrogen peroxide was diluted with H2O, and its concentration was determined at 240 nm using the extinction coefficient 39.4 m−1 cm−1 (16). H2O2 stock solutions were prepared freshly before the experiments. Cyanide, bromide, tyrosine, and all other chemicals were also purchased from Sigma-Aldrich at the highest grade available.

Stopped-flow Spectroscopy—Reactions of LspPOX or LspPOX (H2O2) with cyanide or H2O2 were measured in the conventional stopped-flow mode by following the decrease of absorbance at 412 nm (Soret maximum of ferric LspPOX) or 413 nm (Soret maximum of LspPOX (H2O2)) or at 431/432 nm (Soret bands of cyanide complex of LspPOX and LspPOX (H2O2)). In a typical experiment, one syringe contained 4 μM LspPOX or LspPOX (H2O2), and the second syringe contained at least 10 times molar excess of cyanide or hydrogen peroxide. All reactions were performed in 100 mM phosphate buffer, pH 7.0, at 25 °C. Three determinations were performed for each ligand or oxidant concentration. The mean of the pseudo-first-order rate constants, kobs, was used in the calculation of the second-order rate constants obtained from the slope of a plot of kobs versus ligand or oxidant concentration.

Because of the inherent instability of compound I of LspPOX and LspPOX (H2O2), the sequential stopped-flow (multi-mixing) technique was used for determination of rates of the reaction of compound I with one- and two-electron donors. Similar to bovine LPO, LspPOX (H2O2) compound I could be formed with equimolar concentrations of H2O2, whereas full compound I of LspPOX could only be generated with 6.7 times molar excess of cyanide or hydrogen peroxide. In a typical experiment, 4 μM of LspPOX (H2O2) or LspPOX were premixed with 4 μM or 26.8 μM H2O2 in the aging loop. After 100 ms, the formed compound I was mixed with increasing concentrations of various electron donors. Direct compound I reduction to the ferric state by halides was followed by monitoring of the absorbance change at 412 nm for LspPOX and at 413 nm for LspPOX (H2O2). Formation and reduction of compound II by tyrosine was investigated by following the biphasic reaction (at 432 nm) of compound I to compound II and further to the ferric state for both LspPOX and LspPOX (H2O2). All measurements were done in 100 mM phosphate buffer, pH 7.0, at 25 °C with a minimum of three repeats.

All spectral experiments were carried out with the stopped-flow apparatus SX.18MV and Pi-star-180 from Applied Photophysics in either conventional or sequential mode. The optical quartz cell with a path length of 10 mm had a volume of 20 μl. The fastest time for mixing two solutions and recording the first data point was 1 ms. All reactions were followed both at single wavelengths as well as by using a diode array detector. Polychromatic data were analyzed with Pro-Kineticist software from Applied Photophysics. The program simultaneously fits the kinetic traces at all wavelengths to the proposed reaction mechanism and simulates the spectra of all reactants, products, and intermediate species as well as their time-dependent distribution on the reaction coordinates. Monochromatic data were analyzed and fitted with Pro-Data Viewer software (Applied Photophysics) to calculate time-dependent rate constants.

Experimental Procedures

Materials—Cloning, expression and purification of LspPOX were described in detail previously (13). LspPOX had a purity index (A412/A280) of ~0.7. The ferric high-spin LspPOX and LspPOX pretreated with 15 times molar excess of hydrogen peroxide, i.e. LspPOX (H2O2), had a molar extinction coefficient at Soret maximum of ε412 nm = 94,670 M−1 cm−1 (13).

During the reaction with hydrogen peroxide forming compound I (i.e. oxoiron(IV) porphyrin π-cation radical) (Reaction 2). We investigated compound I reduction by the two-electron donors (X−) bromide, iodide, and thiocyanate to the corresponding (hypo)halous acids (Reaction 3) as well as all reactions involved in the peroxidase cycle, including compound I (Reaction 4) and compound II (i.e. oxoiron(IV)) reduction by one-electron donors (AH2), thereby releasing radical species (●AH) (Reaction 5). Furthermore, we monitored whether compound I reacts with H2O2 (Reaction 6), which is observed with MPO (14, 15).

We report the kinetics of Reactions 1–6 of both LspPOX and LspPOX (H2O2) and demonstrate the significant impact of this PTM on the individual reaction steps. The reported apparent bimolecular rate constants are compared with those known from the mammalian counterparts and discussed with respect to the known structures of MPO (3–5) and LPO (6).

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RESULTS

Cyanide Binding—Fig. 2A shows the spectral changes upon addition of 100 μM or 10 mM cyanide to 2 μM recombinant peroxidase from the cyanobacterium Lyngbya sp. PCC 8106 (LspPOX). Following Reaction 1, the ferric protein purified from *E. coli* was converted from the high-spin (*S* = 5/2) iron state to the low-spin (*S* = 1/2) state, thereby shifting the Soret peak from 412 nm to 431 nm with a clear isosbestic point at 421 nm. Concomitantly, in the visible range a new peak at 547 nm appeared. As depicted in Fig. 2B, the time traces were markedly biphasic with a rapid first phase and a dominating slower second phase. The corresponding pseudo-first-order rate constants, *k*<sub>obs(1)</sub> and *k*<sub>obs(2)</sub>, could be obtained from double-exponential fits. From the corresponding plots of *k*<sub>obs</sub> versus cyanide concentration (*k*<sub>obs</sub> = *k*<sub>on</sub> [CN<sup>-</sup>] + *k*<sub>off</sub>) (Fig. 2, C and D), apparent second-order rate constants of (2.13 ± 0.06) × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup> (K<sub>D</sub> = *k*<sub>off</sub>/*k*<sub>on</sub> = 9.2 μM) and (6.0 ± 0.37) × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup> (K<sub>D</sub> = 35 μM) could be obtained (25 °C).

Interestingly, when LspPOX was incubated with 15 times stoichiometric excess of hydrogen peroxide for 1 h at room temperature (in the following designated as LspPOX (H<sub>2</sub>O<sub>2</sub>)), the kinetics of cyanide binding changed. Ferric LspPOX (H<sub>2</sub>O<sub>2</sub>) exhibits a Soret maximum at 413 nm and two additional peaks at 502 nm and 638 nm. Recently, spectral differences between LspPOX and LspPOX (H<sub>2</sub>O<sub>2</sub>) could be assigned by mass spectrometry to differences in the extent of heme to protein linkages, ranging from only 20% in the recombinant protein purified from *E. coli* to >70% in LspPOX (H<sub>2</sub>O<sub>2</sub>). Cyanide binding in the latter (low-spin complex with maxima at 432 nm and 547 nm and an isosbestic point at 423 nm) was also biphasic but now with a dominating fast phase (being responsible for ~85% of the absorbance change at 432 nm) followed by a second slower phase. The corresponding pseudo-first-order rate constants, *k*<sub>obs(1)</sub> and *k*<sub>obs(2)</sub>, could be obtained from double-exponential fits and allowed the calculation of the apparent second-order rate constants to be (2.06 ± 0.02) × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup> (K<sub>D</sub> = 7.9 μM) (Fig. 3C) and (4.6 ± 0.26) × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup> (K<sub>D</sub> = 22.1 μM) (Fig. 3D), respectively, at pH 7.0 and 25 °C (Table 1).

In both, LspPOX and LspPOX (H<sub>2</sub>O<sub>2</sub>), cyanide binding was biphasic with a fast reaction followed a slower one. Both proteins exhibited almost identical rate constants for these two phases, suggesting that the slow reaction reflects cyanide binding to the heme β protein, whereas the fast reaction reflects cyanide binding to the protein modified by PTM. This is underlined by the fact that in LspPOX, the slower binding phase dominated, whereas in LspPOX (H<sub>2</sub>O<sub>2</sub>), the fast reaction was responsible for the large part of absorbance change. Moreover, the apparent bimolecular rate constant as well as the dissociation constant of the fast cyanide binding phase in the bacterial peroxidase are comparable with those of MPO (1.3 × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup>, K<sub>D</sub> = 4.3 μM) (17) and LPO (1.3 × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup>, K<sub>D</sub> = 23.8 μM) (18) at pH 7.0 and 25 °C.

Compound I Formation—Based on these significant differences in cyanide binding between LspPOX and LspPOX (H<sub>2</sub>O<sub>2</sub>), we further investigated the redox reactions between the respective high-spin ferric proteins and hydrogen peroxide (Reaction 2). With LspPOX, 6.7 times molar excess of H<sub>2</sub>O<sub>2</sub> was necessary to obtain maximum hypochromicity at the Soret maximum (412 nm). An additional prominent peak of LspPOX compound I was observed at 663 nm (black spectrum in boldface in Fig. 4A). Similar to cyanide binding, the kinetics of compound I formation showed a pronounced biphasicity with a rapid single exponential phase (k<sub>obs(1)</sub>) responsible for ~15% absorbance change at 412 nm followed by a slower single exponential phase (k<sub>obs(2)</sub>) responsible for ~85% absorbance change at the Soret maximum.

Compound I of LspPOX (H<sub>2</sub>O<sub>2</sub>) was characterized by a 45% decrease of absorbance at the Soret band (Fig. 5A). Already equimolar H<sub>2</sub>O<sub>2</sub> was sufficient to reach the full hypochromicity at the Soret maximum (413 nm), very similar to EPO (20) and
Upon compound I formation, absorbance at 502 and 638 nm was lost, and a new peak at 663 nm appeared (black spectrum in Fig. 5A). Compound I formation of LspPOX(H₂O₂) was still biphasic but now showed a dominating rapid exponential decrease at 413 nm (k_{obs(1)}, responsible for 85% absorbance change), followed by a slower decrease (k_{obs(2)}) at the Soret maximum (Fig. 5B).

Upon calculation of the apparent bimolecular rate constant of the initial rapid phase (i.e. k_{obs(1)} values) of compound I formation (Figs. 4E and 5E), similar apparent bimolecular rate constants could be obtained for both metalloproteins, namely (5.2 ± 0.24) × 10⁷ M⁻¹ s⁻¹ for LspPOX and (4.4 ± 0.15) × 10⁷ M⁻¹ s⁻¹ for LspPOX(H₂O₂). Moreover, the apparent second-order rate constants calculated from the k_{obs(2)} values (Figs. 4F and 5F) were almost identical, i.e. (1.7 ± 0.07) × 10⁶ M⁻¹ s⁻¹ and (1.6 ± 0.17) × 10⁶ M⁻¹ s⁻¹, respectively. Together with the correlation between absorbance changes and k_{obs} values of the fast and slower phase in the respective proteins (see above), these data nicely reflect that (i) in LspPOX(H₂O₂) compound I formation is 30 times faster compared with LspPOX and that (ii) ~85% of LspPOX(H₂O₂) contains covalently bound heme, which reflects the mass spectrometric findings reported recently (13). The bimolecular rate constant of LspPOX(H₂O₂) is faster compared with MPO (14, 15) and LPO (21) (Table 1).

Besides the discrepancy in the apparent bimolecular rate constants between the heme b protein and the posttranslationally modified peroxidase, the corresponding plots also showed differences in y axis intercepts (compare Figs. 4E and 5E with Figs. 4F and 5F), which might reflect differences in (i) H₂O₂ binding (e.g. faster dissociation from the initial complex between the protein and the peroxide in the modified peroxidase) and/or in (ii) reactivity and stability of the resulting compound I. As will be shown below, compound I of LspPOX(H₂O₂) is a stronger oxidant of bromide compared with that of LspPOX. The increased reactivity is also reflected by a more positive reduction potential of the Fe(III)/Fe(II) couple of LspPOX(H₂O₂) (13).

With both proteins, compound I was not stable. In case of LspPOX(H₂O₂), compound I decayed (Fig. 5C) directly to an intermediate with spectral features very similar to those of an intermediate in compound I.
oxoiron(IV) species (Fig. 5A, light gray spectra) with maxima at 432, 538, and 564 nm (rate of decay of ~3 s−1). After adding a 6.7 times molar excess of H2O2 to LspPOX, it took at least 100 ms to reach full hypochromicity at the Soret absorbance, but the resulting compound I also decayed (Fig. 4C) to an intermediate with spectral features identical to those of LspPOX (H2O2) (Fig. 4A, gray spectra). In both cases, formation of this species with compound II-like spectral properties did not depend on the concentration of hydrogen peroxide (Figs. 4D and 5D). Formation of an oxoiron(IV) protein radical species in the absence of exogenous electron donors has also been demonstrated with LPO and EPO (19). Only in MPO, compound I can be reduced with hydrogen peroxide to compound II with a
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bimolecular rate-constant of $4.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ according to Reaction 6 (Table 1) (14, 15). Oxidation of $\text{H}_2\text{O}_2$ to superoxide by compound I is typical for myeloperoxidase (14, 15), but neither LspPOX ($\text{H}_2\text{O}_2$) nor EPO (20) or LPO (21) can catalyze this reaction.

**Reaction of Compound I with Two-electron Donors**—Next, we studied the effect of the posttranslational modification on the reactivity of compound I with halides and thiocyanate according to Reaction 3. In detail, we premixed LspPOX with 6.7 times excess $\text{H}_2\text{O}_2$ and LspPOX ($\text{H}_2\text{O}_2$) with equimolar $\text{H}_2\text{O}_2$ in the aging loop for 100 ms to get a maximum of compound I. For both species, this guaranteed a minimum decay of compound I. With chloride (up to 100 mM) as electron donor, no reaction occurred, and compound I of both proteins decayed to the oxoiron(IV) species described above.

Upon mixing compound I of LspPOX with bromide, formation of this compound II-like species was suppressed to some extent and the absorbance at the Soret maximum (412 nm) increased, indicating the occurrence of a direct transition of compound I back to ferric LspPOX (Fig. 6A). The higher the bromide concentration, the more negligible was the decay of compound I to the oxoiron(IV) species (shoulder at 432 nm). Fig. 6B depicts typical time traces at 412 and 432 nm for the reaction with 50 $\mu$M bromide. The observed initial steady-state phase is consistent with the presence of excess of hydrogen peroxide that had to be used for compound I formation of LspPOX. Ferric enzyme produced by reaction of compound I with bromide was immediately reoxidized by excess of $\text{H}_2\text{O}_2$ to compound I, which could react again with bromide or decay to the oxoiron(IV) species. The higher the bromide concentration, the faster $\text{H}_2\text{O}_2$ was consumed and the shorter was this initial steady-state phase. The increase of absorbance at 412 nm (after this initial phase) could be fitted with a double-exponential function (Fig. 6B). The slope of the plot of pseudo-first-order rate constant, $k_{\text{obs}(1)}$, against bromide concentration gave an apparent bimolecular rate constant of $(4.1 \pm 0.3) \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ at pH 7.0 and 25 °C (Fig. 6C). With iodide and thiocyanate, similar time traces were observed, and apparent bimolecular rate constants could be calculated to be $(5.3 \pm 0.32) \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ (iodide) and $(5.3 \pm 0.57) \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ (thiocyanate), respectively (Fig. 6, D and E). The $y$-intercepts of the plots $(2.0–4.4 \text{ s}^{-1})$ might reflect the instability and decay of compound I as described above.

By contrast, with LspPOX ($\text{H}_2\text{O}_2$) an almost direct transition of compound I (built with equimolar $\text{H}_2\text{O}_2$) to ferric LspPOX ($\text{H}_2\text{O}_2$) was observed (Fig. 7A). Typical time traces at 413 nm still showed a double-exponential character (Fig. 7B) with the first fast phase of the reaction being strongly dependent on the halide concentration (Fig. 7C), whereas the second slower part being independent of the halide concentration and reflecting the formation of the oxoiron(IV) species. In Fig. 7C, the apparent second-order rate constant was calculated from the slope of the linear plot of pseudo-first-order rate constant, $k_{\text{obs}(1)}$, against bromide concentration to be $(1.8 \pm 0.05) \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, which is about four times faster compared with LspPOX and LPO (21) but slower compared with MPO (22) (Table 1). With iodide and thiocyanate similar spectral transitions were obtained. The impact of the instability of compound I was significantly smaller due to the higher reactivity with both iodide and thiocyanate (Fig. 7, D and E). Inspection of the corresponding plots reveals a higher intercept compared with those for LspPOX, which could reflect differences in iodide or thiocyanate binding to compound I in these two species. In any case, the reactions between iodide or thiocyanate and compound I of LspPOX ($\text{H}_2\text{O}_2$) were extremely fast, i.e. $(4.2 \pm 0.27) \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ (iodide) and $(2.0 \pm 0.07) \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ (thiocyanate). The big differences in reaction rates between

**FIGURE 6.** Reaction of LspPOX compound I with (pseudo-)halides. A, spectral transition after addition of 50 $\mu$M bromide to LspPOX compound I (2 $\mu$M) in the sequential mixing stopped-flow mode. Compound I was preformed with 6.7 times excess of $\text{H}_2\text{O}_2$ (red) as described under “Experimental Procedures.” After a 100-ms delay, the first spectrum was recorded at 2 ms, and subsequent spectra were recorded at 101 ms, 251 ms, 406 ms, 551 ms, 707 ms, 860 ms, 1.04 s, 1.4 s, 2.03 s, 3.1 s, 5 s. The reaction was carried out in 100 mM phosphate buffer, pH 7.0, 25 °C with a 100-ms delay time. B, typical time traces at 412 nm and 432 nm as well as a double-exponential fit (red) of the reaction of LspPOX compound I with 50 $\mu$M bromide followed at 412 nm. Conditions were as described in A. C, pseudo-first-order rate constants $k_{\text{obs}}$ (red) plotted against bromide concentration. Final conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, with a 100-ms delay time. D, pseudo-first-order rate constants $k_{\text{obs}}$ (green) plotted against iodide concentration. Final conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, 1 $\mu$M LspPOX and 67 $\mu$M $\text{H}_2\text{O}_2$, with a 100-ms delay time. E, pseudo-first-order rate constants $k_{\text{obs}}$ (blue) plotted against thiocyanate concentration. Final conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, 1 $\mu$M LspPOX and 67 $\mu$M $\text{H}_2\text{O}_2$, with a 100-ms delay time.
LspPOX and LspPOX (H₂O₂) are obvious, i.e., 80 times (iodide) or 345 times (thiocyanate) higher rates in LspPOX (H₂O₂) (Table 1). Iodide and thiocyanate oxidation by LspPOX (H₂O₂) is faster than with MPO compound I (22) but slower compared with the LPO intermediate (21).

Reactions between Compound I and Compound II and Tyrosine—Finally, we analyzed compound I (Reaction 4) and compound II reduction (Reaction 5) of both proteins by tyrosine. Upon addition of tyrosine to LspPOX compound I (Fig. 8A, red line), compound II (black line) was formed, and after a distinct steady-state phase (caused by excess of hydrogen peroxide in the system), compound II was converted to the ferric protein. The length of this steady-state phase depended on the tyrosine concentration. The corresponding time traces at 412 and 432 nm (i.e. Soret maximum of compound II) are shown in Fig. 8B. From the time traces at 432 nm, pseudo-first-order rate constants $k_{obs}$ were plotted against tyrosine concentrations. Conditions were as described in C.

FIGURE 7. Reaction of LspPOX (H₂O₂) compound I with (pseudo-)halides. A, spectral transition after addition of 10 μM bromide to LspPOX (H₂O₂) compound I (2 μM) in the sequential mixing stopped-flow mode. Compound I was preformed with equimolar H₂O₂ as described under “Experimental Procedures.” After a 100-ms delay, the first spectrum was recorded at 2 ms, and subsequent spectra were recorded at 37 ms, 123 ms, 212 ms, 347 ms, 609 ms, 1.77 s, and 5 s. The reaction was carried out in 100 mM phosphate buffer, pH 7.0, 25 °C, with a 100-ms delay time. B, typical biphasic time trace and double-exponential fit (red) of the reaction of LspPOX (H₂O₂) compound I with 10 μM bromide followed at 413 nm. Conditions were as described in A. C, pseudo-first-order rate constants $k_{obs}$ (red) plotted against bromide concentration. Final conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, 1 μM LspPOX (H₂O₂), 1 μM H₂O₂, with a 100-ms delay time. D, pseudo-first-order rate constants $k_{obs}$ (green) plotted against iodide concentration. Final conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, 0.6 μM LspPOX (H₂O₂), 1 μM H₂O₂, with a 100-ms delay time. E, pseudo-first-order rate constants $k_{obs}$ (blue) plotted against thiocyanate concentration. Final conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, 0.6 μM LspPOX (H₂O₂), 1 μM H₂O₂, with a 100-ms delay time.

FIGURE 8. Reduction of LspPOX compound I and compound II by tyrosine. A, spectral changes upon addition of 50 μM tyrosine to 2 μM compound I in the sequential mixing stopped-flow mode. Compound I was formed with ferric LspPOX and 6.7 times excess of H₂O₂ (red). After a 100-ms delay, the first spectrum was recorded at 5 ms, and subsequent spectra were recorded at 100 ms, 298 ms, 900 ms, 2.4 s (compound II-like, black labeled) and 3.2 s, 3.5 s, 3.9 s, and 10 s. The inset to Fig. 8A shows the spectral transition in the visible range. Final reaction conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, with a delay time of 100 ms. B, typical time traces at 432 nm, the maximum absorbance of compound II and 412 nm, the maximum absorbance of ferric LspPOX. Conditions were as described in A. C, multiphasic time trace at 432 nm with both single-exponential fits, (i) the initial phase of compound II formation (red) and (ii) the last phase of compound II reduction (green). Final conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, 1 μM LspPOX and 6.7 μM H₂O₂, 100-ms delay time, 50 μM tyrosine. D and E, pseudo-first-order rate constants for LspPOX compound I reduction (red) at 432 nm (D) and (in green at 432 nm) compound II reduction (E) both plotted against tyrosine concentrations. Conditions were as described in C.
constants of compound II formation and reduction were obtained by fitting the respected phases by single-exponential equations (Fig. 8C). Apparent second-order rate constants were calculated from the corresponding linear plots (Fig. 8, D and E) to be (3.2 ± 0.17) × 10^4 M⁻¹ s⁻¹ (compound I reduction) and (5.5 ± 0.27) × 10^4 M⁻¹ s⁻¹ (compound II reduction) (Table 1).

Upon addition of tyrosine to LspPOX (H₂O₂) compound I (red line), sequential formation of compound II (black line) and its reduction was observed (Fig. 9A). Transition of compound II to compound II showed a defined isosbestic point at 413 nm, whereas the transition of compound II to ferric LspPOX (H₂O₂) exhibited an isosbestic point at 425 nm. Fig. 9B shows a typical biphasic time trace observed at 432 nm, which was obtained when LspPOX (H₂O₂) compound I was mixed with the one-electron donor tyrosine. After initial formation of compound II (Soret maximum at 432 nm), the reaction continues and ferric LspPOX (H₂O₂) was formed (decrease at 432 nm and increase at 413 nm). Both parts of the time trace could be fitted with a single-exponential function (Fig. 9C), and the apparent second-order rate constants were determined to be (6.2 ± 0.15) × 10^5 M⁻¹ s⁻¹ for compound I reduction and (1.1 ± 0.07) × 10^5 M⁻¹ s⁻¹ for compound II reduction (Fig. 9, D and E). Both rate constants are similar or even higher than those reported for MPO (23) or LPO (Table 1) (24).

**DISCUSSION**

Heme proteins conduct a myriad of different biological functions, including oxygen transport, storage and reduction, electron transfer, and redox catalysis. Thus, it is challenging to investigate and understand the role of (posttranslational) modifications of the prosthetic group and/or of its protein environment in fine-tuning of biophysical and biochemical features of these oxidoreductases. In the majority of heme peroxidase families unmodified heme b (e.g. in horseradish peroxidase (HRP) (Fig. 1A)) is found at the active site (Fig. 1B). So far, prominent exceptions included vertebrate (including human) peroxidases that are highly homologous to LPO in cyanobacterium Lyngbya sp. PCC 8106 corresponds to LspPOX (H₂O₂). The cyanobacterium Lyngbya sp. accommodates an oxygenic photosynthetic together with a respiratory electron transport chain within a single prokaryotic (non-compartmentalized) cell, thereby performing oxygen activation and generation of hydrogen peroxide at high rates.

Most likely via H₂O₂-mediated oxidation and compound I formation, the nearby located carboxyl side chains of Asp-100 and Glu-229 were oxidatively attacked, finally leading to ester bond formation as described for its mammalian counterparts.

![Figure 9](image-url)

**FIGURE 9. Reduction of LspPOX (H₂O₂) compound I and compound II by tyrosine.** A, spectral changes upon addition of 10 μM tyrosine to 2 μM compound I in the sequential mixing stopped-flow mode. Compound I was formed with ferric LspPOX (H₂O₂) and equimolar H₂O₂ (red). With a delay time of 100 ms, the first spectrum was recorded at 5 ms, and subsequent spectra were recorded at 100 ms, 216 ms, 450 ms, 900 ms (compound II-like, black labeled) and 1.3 s, 1.6 s, 1.9 s, 2.7 s, and 10 s. The inset shows the spectral transition in the visible range. B, pseudo-first-order rate constants for LspPOX (H₂O₂) compound I. C, pseudo-first-order rate constants for LspPOX (H₂O₂) compound II. D, pseudo-first-order rate constants for LspPOX (H₂O₂) compound II. E, the second phase of compound II reduction (green) and the second phase of compound II reduction (green) in the visible range. Final reaction conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, with a delay time of 100 ms. Typical time traces at 432 nm, the maximum absorbance of compound II and 413 nm, the maximum absorbance of ferric LspPOX (H₂O₂), Conditions were as described in A, C, biphasic time trace at 432 nm with both single-exponential fits, (i) for the initial phase of compound II formation (red) and (ii) the second phase of compound II reduction (green). Conditions were as described in A, C, biphasic time trace at 432 nm with both single-exponential fits, (i) for the initial phase of compound II formation (red) and (ii) the second phase of compound II reduction (green). Final reaction conditions were 100 mM phosphate buffer, pH 7.0, 25 °C, 1 μM LspPOX (H₂O₂), and equimolar H₂O₂, 100-ms delay time, 10 μM tyrosine. D and E, pseudo-first-order rate constants for LspPOX (H₂O₂) compound I. D, pseudo-first-order rate constants for LspPOX (H₂O₂) compound II (red) at 432 nm (D) and (in green at 432 nm) compound II reduction (E) both plotted against tyrosine concentrations. Conditions were as described in C.
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(7–9). Thereby, most likely the symmetry of the heme group as well as its planarity were lowered as was evident by distinct changes of the UV-visible electronic absorption, circular dichroism, and electronic paramagnetic resonance spectra as described recently (13). It was interesting to see that during this autocatalytic process the spectral properties of the ferric and ferrous forms of the bacterial peroxidase became very similar to LPO. Moreover, the standard reduction potential \( (E^\circ) \) of the Fe(III)/Fe(II) couple (−145 mV) became similar to that of LPO (−176 mV) (25) and EPO (−126 mV) (25). This result compares with reported \( E^\circ \) values of −190 mV and −306 mV for the heme \( b \) proteins cytochrome \( c \) peroxidase and horseradish peroxidase (26). It reflects the impact of heme to protein ester bonds on heme distortion by promoting the out of plane displacement of the heme iron in mammalian peroxidases (6, 25, 26) and most probably also in ferric LspPOX (\( H_2O_2 \)).

The possibility to monitor the peroxide driven transition from a predominantly heme \( b \) in LspPOX to mainly modified and covalently linked heme in LspPOX (\( H_2O_2 \)) motivated us to study the impact of this PTM on the kinetics of the formation and reaction of all relevant redox intermediates of the halogenation and peroxidase cycles. For the mammalian counterparts (e.g. MPO or LPO), this could not be followed so far (despite the presence of comparable inhomogeneities regarding the covalent heme to protein bonds both in MPO or LPO).

Upon incubation of recombinant ferric LspPOX with hydrogen peroxide both cyanide binding and \( H_2O_2 \)-mediated oxidation to compound I was accelerated, and the \( k_{app} \) value for cyanide was decreased. It has to be mentioned that in regard to the rate of compound I formation, there are no obvious differences between peroxidases with and without covalently linked heme. The \( k_{app} \) values of this bimolecular reaction in mammalian peroxidases as well as plant-type peroxidases (such as horseradish peroxidase) are within \( (1.1–5.6) \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) at pH 7.0 (1, 2). This result clearly suggests that upon transition from LspPOX to LspPOX (\( H_2O_2 \)), changes of both (i) the structure of the distal heme cavity as well as of (ii) the access channel to the active site occurred. Both cyanide and hydrogen peroxide binding to the ferric state need the distal (catalytic) histidine to act as proton acceptor before the resulting anions bind to the ferric heme. Additionally, in heme peroxidases the distal histidine is involved in hydrogen bonding of the resulting cyanide complexes as well as of higher oxidation states such as compound II (1, 4). Modification of the prosthetic group and formation of covalent bonds with the protein will have an impact on these interactions. Moreover, because the substrate channel opens into the heme cavity close to the \( \delta \)-meso bridge between pyrrole rings A and D (Fig. 1), these PTMs will also affect the accessibility of ligands and substrates. This result has been demonstrated for MPO variants, where the exchange of this glutamate residue (i.e. Glu-242) significantly reduced the rate of cyanide binding and compound I formation, most likely because the flexible side chain blocked the entrance to the heme cavity (27, 28). A similar scenario supposedly accounts for the lower reactivity in LspPOX with mainly unmodified glutamate.

Compound I of LspPOX (\( H_2O_2 \)) could be formed with equimolar concentration of hydrogen peroxide. This fact as well as the spectral features of the resulting intermediate are very similar to LPO compound I (1, 24). In the absence of exogenous electron donors, the protein moiety of the bacterial protein is oxidized resulting in an oxoiron(IV) intermediate with a compound II-like spectrum. This effect is also observed with LPO and reflects the similar redox properties of the two enzymes (13, 19, 25).

The oxidation capacity of compound I of LspPOX was significantly increased upon PTM. Similar to LPO, chloride could not act as electron donor in LspPOX (\( H_2O_2 \)). Chloride oxidation is known from MPO and closely related to the existence of its electron-withdrawing sulfonium ion linkage and its strongly distorted and asymmetric heme group (3–5, 29), which results in the extraordinary positive reduction potential of its Fe(III)/Fe(II) couple (Table 1) (11, 27). By contrast, bromide acts as two-electron donor of compound I of LspPOX (\( H_2O_2 \)) and the calculated apparent bimolecular rate constant was even higher compared with LPO compound I, which nicely reflects the recently published steady-state bromination data (13) but also the slightly more positive reduction potential of the Fe(III)/Fe(II) couple of LspPOX (\( H_2O_2 \)). Although not directly involved in the catalytic cycle, the hierarchy of reduction potentials of the Fe(III)/Fe(II) couple often reflects the hierarchy of the reduction potential \( (E^\circ) \) of the compound I/Fe(III) couple. Based on this, the hierarchy of \( E^\circ \) (compound I/Fe(III)) is suggested to be MPO > EPO > LspPOX (\( H_2O_2 \)) > LPO (11, 13).

An interesting observation was also the drastic increase of apparent bimolecular rate constants of the reactions of compound I with iodide and thiocyanate after PTM. This cannot be related with the increase of the oxidation capacity of the bacterial peroxidase as oxidation of these two-electron donors is not challenging thermodynamically (30). As already discussed above, probably in LspPOX, the negatively charged and flexible glutamate impedes the accessibility of these (bigger) anionic electron donors. Upon PTM and covalent bond formation, the negative charge disappears and additionally (most likely) the accessibility to the active site is increased (27–29).

Finally, we could demonstrate that the PTM also modulated the peroxidase cycle of this novel bacterial peroxidase (Reactions 2, 4, and 5). Both relevant redox intermediates, namely compound I and compound II, exhibited increased oxidation rates of tyrosine after the autocatalytic transformation (Table 1). Importantly, the observed UV-visible spectral features of compound II are also similar to LPO and significantly different to heme \( b \) peroxidases. With tyrosine \( (E^\circ = 0.93 \text{ V} (31)) \) as model substrate, it was demonstrated that the apparent bimolecular rate constants of both compound I reduction (Reaction 4) and compound II reduction (Reaction 5) of LspPOX (\( H_2O_2 \)) were about two times higher compared with LspPOX (Table 1). Both compound I and compound II of LspPOX (\( H_2O_2 \)) are stronger oxidants compared with the corresponding redox intermediates of lactoperoxidase (Table 1) and horseradish peroxidase (32). In both horseradish peroxidase and mammalian peroxidases, the oxidation of aromatic electron donors has been proposed to take place in the vicinity of the \( \delta \)-mesocarbon and pyrrole ring D (compare Fig. 1, B, D, and F) (1, 32). Based on the sequence homology of the bacterial protein with LPO (13), it is reasonable to assume that the oxidation site of tyrosine in LspPOX (\( H_2O_2 \)) is very similar. This, together with the hierar-
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of the reduction potential of the Fe(III)/Fe(II) couples of these peroxidases (11, 13, 25, 32), suggests that the $E^\prime\prime$ values of the couples compound I/compound II as well as compound II/Fe(III) follow the hierarchy LspPOX (H$_2$O$_2$) $>\$ LPO $>\$ HP$. It demonstrates that the bacterial peroxidase is an efficient catalyst of both two- and one-electron oxidation reactions. These properties together with its high conformational and thermal stability (13) render this novel class of heme proteins very interesting for application as catalyst in biotechnology.

Summing up, for the first time a recombinant heme peroxidase allowed monitoring of the effect of hydrogen peroxide-driven formation of covalent heme to protein bonds on its biochemical and biophysical properties. Distinct changes in spectral and redox properties during this structural transition were observed (13). This posttranslational modification alters the architecture of the heme cavity thereby improving the binding of cyanide and H$_2$O$_2$. Upon covalent bond formation, the reduction potentials of the relevant redox intermediates and their reactivity toward one- and two-electron donors are increased. Additionally, the accessibility to the (pseudo-)halide binding and oxidation site seems to be increased.

It has to be mentioned that the physiological role of these bacterial heme peroxidases is completely unknown so far. Lsp-POX is the first studied representative of a novel peroxidase family, which can be regarded as phylogenetic origin in the evolution of the vertebrate peroxidases, including LPO and MPO (13). By sharing structural, biophysical and catalytic properties, one might speculate whether these bacterial proteins also share similar biological roles, i.e. unspecific defense reactions by production of halogenating and antimicrobial products such as hypohalous acids.

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