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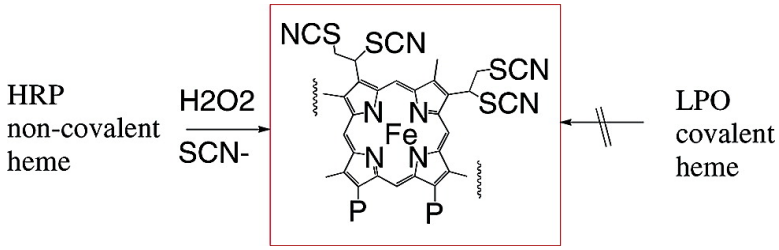

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Autocatalytic Modification of the Prosthetic Heme of Horseradish but Not Lactoperoxidase by Thiocyanate Oxidation Products. A Role for Heme−Protein Covalent Cross-Linking

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Abstract: The mammalian peroxidases eosinophil peroxidase, lactoperoxidase (LPO), and myeloperoxidase oxidize thiocyanate to the antimicrobial agents hypothiocyanous acid (HOSCN) and $(SCN)_2$ and are part of a defense system that protects the host from infections. Horseradish peroxidase (HRP), a plant enzyme, also oxidizes thiocyanate. We report here that the prosthetic heme vinyl groups of HRP react with the catalytically generated HOSCN and $(SCN)_2$ to form at least nine vinyl-modified heme adducts. Mass spectrometry combined with analysis of the equivalent reactions of HRP reconstituted with 2- or 4-cyclopropylheme, or mesoheme-d_4, shows that all of the prosthetic heme modifications result from addition of oxidized thiocyanate to the heme vinyl groups. No δ-*meso*-substitution of the heme was observed, in contrast to what is observed with radical agents. Model studies show that incubation of either HRP with preformed HOSCN or a solution of heme with preformed $(SCN)_2$ gives rise to the same products obtained in the HRP-catalyzed reaction. Model studies also demonstrate that the SCN• radical, if formed, should add to a *meso*-carbon. These findings implicate an electrophilic addition mechanism. In contrast, oxidation by LPO of thiocyanate, the normal substrate of this enzyme, does not result in heme modification. In view of the demonstrated intrinsic reactivity of the heme group, LPO must actively suppress heme modification. As the key difference between LPO (and other mammalian peroxidases) and HRP is the presence of two covalent ester links between the heme and the protein, we propose that these links contribute to steric protection of the adjacent heme vinyl groups.

Introduction

The mammalian peroxidases, which include myeloperoxidase (MPO), lactoperoxidase (LPO), and eosinophil peroxidase, catalyze the H_2O_2-dependent oxidation of halide ions and pseudohalides such as thiocyanate (SCN^-) to reactive species whose chemical reactivity affords antimicrobial protection.[1−4] Although MPO is the only one of these enzymes for which a crystal structure is available,[5−7] it is now clear that they are unique among the hundreds or thousands of known peroxidases in that the prosthetic heme group in the mammalian peroxidases is covalently bound to the protein. In LPO, for which covalent heme binding has been clearly established, the heme is linked to the protein via ester bonds between Glu375 and the heme 1-methyl and Asp225 and the heme 5-methyl.[8,9] The same two methyl groups of the heme of MPO are similarly bound to the protein, but in addition MPO has a third covalent link between the sulfur of Met243 and the terminal carbon of the 2-vinyl group.[6] This third link places a positively charged sulfur substituent on the vinyl group. Crystal structures have shown that in MPO Br^- and SCN^- bind in the active site near the δ-*meso* carbon of the heme,[10] and an NMR study has provided evidence that in LPO SCN^- binds within approximately 7.2 Å of the heme iron, presumably near the δ-*meso* carbon of the porphyrin.[11]

MPO is the only mammalian peroxidase that efficiently oxidizes chloride ion,[12] but the other mammalian enzymes with the exception of thyroid peroxidase readily oxidize bromide, iodide, and thiocyanate.[12−14] Indeed, in the case of LPO, thiocyanate is almost certainly the biologically relevant endog-

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enous substrate,[15] and even for MPO and EPO, comparison of the rates of halide and thiocyanate oxidation suggests that thiocyanate may also be the major physiological substrate for these enzyme.[1,2,16]

Horseradish peroxidase (HRP), a plant enzyme, differs from the mammalian peroxidases in that, like most peroxidases, its prosthetic heme group is not covalently bound to the protein. HRP catalyses the oxidation of a diversity of small inorganic substrates, including nitrite,[17,18] azide,[19,20] iodide,[21,22] bromide,[23] chloride,[23] cyanide,[24] and thiocyanate.[25−28]

The oxidation of thiocyanate by LPO and MPO results in the accumulation of hypothiocyanous acid (HOSCN) and its anion,[16,29−31] whereas the product accumulated in the oxidations catalyzed by HRP appears to be dithiocyanogen $(SCN)_2$.[25,28] Some evidence exists for formation of the radical species NCS• in both the LPO and HRP reactions.[28,32] Nevertheless, hypothiocyanous acid is certain to be present in all of the reactions.

The catalytic oxidation of SCN^- by HRP is 100-fold lower than that by LPO at the optimum pH.[28] Banerjee et al.[28] attributed this slower oxidation rate for HRP than LPO to reversible inactivation of HRP but not LPO during SCN^- oxidation. HRP is inactivated by a pseudo first-order kinetic process with a rate constant of 400 M^{-1},[28] a process that does not appear to occur with LPO. The inactivation was attributed to the formation, at high concentrations of SCN^-, of CN^- that coordinated to the heme iron atom of HRP but not LPO due to hypothetical differences in the dimensions of their active sites.

We report here that the prosthetic heme of HRP is modified at both vinyl groups when the enzyme catalyzes the oxidation of thiocyanate, whereas similar modifications do not occur with LPO. We show, furthermore, that heme in solution reacts with HOSCN and $(SCN)_2$ to give the same modified products. The absence of heme vinyl group modification in LPO by these agents, which are the normal products of LPO in a physiological context, therefore requires that the enzyme active site structure suppress the reactions. We postulate that this is one of the functions of the unique cross-linking of the heme to the protein in the mammalian peroxidases.

Results

Formation of Thiocyanate Heme Adducts. Incubation of HRP with thiocyanate and H_2O_2 in citrate buffer (pH 4, room temperature, 30 min), followed by HPLC analysis with the detector set at 398 nm, reveals the formation of at least nine heme-derived products (Figure 1). The products all exhibit the UV−vis spectra characteristic of a heme chromophore. Mass spectrometric analysis of the products shows that all of them can be accounted for by the addition of from one to four thiocyanate groups, in some instances accompanied by the addition of a hydroxyl group or loss of a proton.

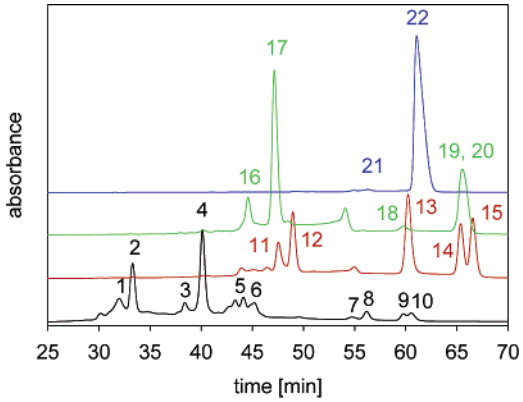

Figure 1. HPLC chromatograms of products from co-incubations of SCN^- and H_2O_2 with HRP containing the indicated prosthetic group: heme (black line), 2-cyclopropylheme (red line), 4-cyclopropylheme (green line), and mesoheme-d_4 (blue line). Absorbances of heme-derived products were recorded at 398 nm.

The primary characterization of the modified hemes has been carried out by mass spectrometry. The mass spectra of compounds **2**, **4**, and **7** are presented in Figure 2 (all of the mass spectra are provided in the Supporting Information). The most polar compound **1** exhibits a molecular ion mass at *m*/*z* 807 consistent with the addition of three thiocyanate groups plus a hydroxyl moiety to the parent heme group [i.e., MW = 616 (heme) + 174 (3 SCN) + 17 (OH)] (Table 1). Compound **2** exhibits an even higher molecular ion at *m*/*z* 848, in agreement with the addition of four thiocyanate groups to the heme. Compound **3** has a molecular ion at *m*/*z* 748, as expected for addition of two thiocyanate groups plus a hydroxyl minus a proton. Compound **4**, with a molecular ion of *m*/*z* 789, has apparently undergone the addition of three thiocyanates with loss of one hydrogen atom. Compound **5**, with a molecular ion at *m*/*z* 732, is a heme to which two thiocyanate groups have been added. Interestingly, compound **6** has the same molecular ion as compound **2** and thus also corresponds to the addition of four thiocyanate moieties to the heme. Compounds **2** and **6** are therefore likely to be isomeric structures. Compound **7** has the molecular ion (*m*/*z* 673) expected for addition of one thiocyanate and loss of one proton. Compound **8** is an isomer of compound **4** in that their molecular ions (and therefore compositions) are identical. Finally, compound **9** has the same molecular ion (*m*/*z* = 732) as compound **5** and is therefore also an isomeric structure (Table 1).

To define the region of the molecule involved in the thiocyanate addition reactions implied by the molecular masses of the products, we reconstituted HRP with mesoheme having a deuterium at each of its *meso*-carbon positions (i.e., mesoheme-d_4) and repeated the incubation with thiocyanate and H_2O_2.

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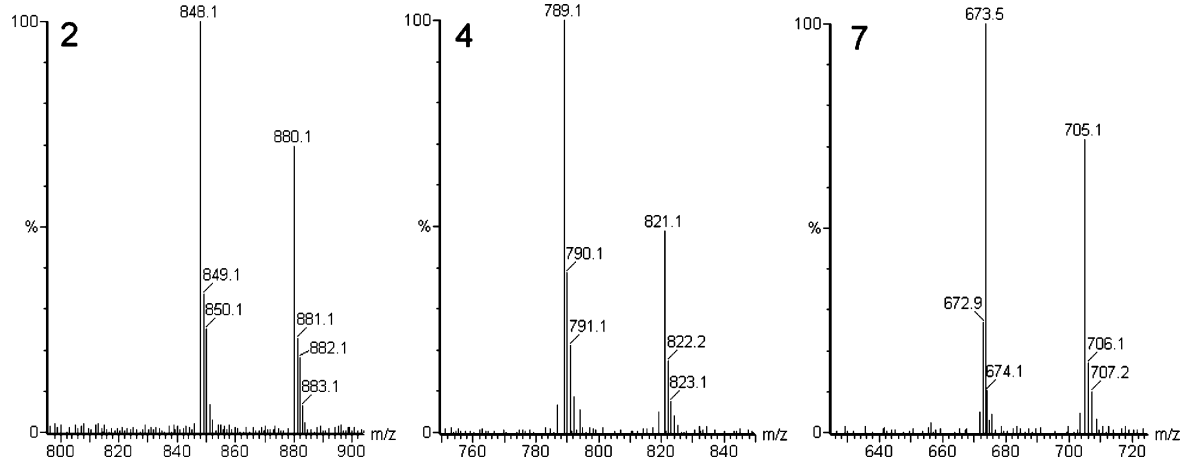

Figure 2. High mass region of the mass spectra of three modified hemes formed upon incubation of HRP with SCN^- and H_2O_2. As labeled, the mass spectra are for products **2**, **4**, and **7** (see Figure 3 for compound structures).

Table 1. Summary of the Mass Spectrometric Data on the Identified Modified Heme Products

compound	M^+ ion (*m*/*z*)[a]	heme	SCN[b] (#)	OH (#)	H	molecular mass
1	807	616	174 (3)	17 (1)		807
2	848	616	232 (4)			848
3	748	616	116 (2)	17 (1)	−1	748
4	789	616	174 (3)		−1	789
5	732	616	116 (2)			732
6	848	616	232 (4)			848
7	673	616	58 (1)		−1	673
8	789	616	174 (3)		−1	789
9	732	616	116 (2)			732
21	680	624[c]	58		−2 (D)	680

[a] The molecular ion is usually accompanied by a strong ion 32 mass unit higher for the methanol-complexed iron porphyrin. [b] The molecular mass of SCN is 58. [c] The mass of *meso*-tetradeuterated mesoheme is 624.

In mesoheme, the 2- and 4-vinyl groups of heme (iron protoporphyrin IX) are replaced by ethyl groups. The molecular mass of *meso*-tetradeuterated mesoheme is 624. The finding (Figure 1, blue line) that none of the modified products are formed in this reaction clearly indicates that heme modification involves reactions of the oxidized thiocyanate with the heme vinyl groups and not with alternative positions such as the *meso*-carbons of the heme. Only a trace (<1%) of a new adduct is observed (hardly detectable at the amplification shown in Figure 1) with a molecular ion corresponding to addition of a thiocyanate with loss of a deuterium, clearly showing that this trace adduct arose by addition of an SCN moiety to one of the four *meso*-carbons bearing the deuterium atoms. Structure **21** (Figure 3, Table 1) is probable for this adduct, as the *δ-meso* position of the heme is the site involved in all of the previously identified heme modification reactions involving a *meso*-carbon of the heme in HRP.[33]

The nine modified hemes observed in the reaction of HRP with thiocyanate and H_2O_2 (Figure 1) thus arise by reactions of oxidized thiocyanate species with the two vinyl groups of the normal prosthetic heme group. Three chemical reactions of HOSCN and $(SCN)_2$, the major species produced from thiocyanate by HRP,[25,28] readily rationalize the observed products. All three reactions are initiated by electrophilic addition of a thiocyanate moiety to a vinyl group, a well-precedented reaction of HOSCN and $(SCN)_2$ that generates a carbocationic intermediate.[34,35] The electrophilic addition is then followed by one of three termination processes: (a) trapping of the resulting carbocation by a thiocyanate anion (Scheme 1, reaction **a**), (b) trapping of the carbocation by a water molecule (Scheme 1, reaction **b**), or (c) loss of a proton to regenerate a double bond (Scheme 1, reaction **c**).

Compound **1** (Figure 3) thus corresponds to modification of one heme vinyl group by reaction **a** and the other by reaction **b**, although the regiochemistry, that is, which vinyl group is involved in each of the two reactions, cannot be specified for this product or the others below without further information. Compound **2** is the result of modification of both vinyl groups by reaction **a**. Compound **3** results from reaction **b** at one vinyl group and reaction **c** at the other, again with an ambiguity concerning which vinyl is involved in each of the two reactions. Compound **4** is explained by reaction a at one vinyl and reaction **c** at the other (Figure 3).

Compounds **5** and **9** involve modification of a single vinyl group by reaction **a**, one being the product of reaction at the 2-vinyl and the other of the 4-vinyl. Compound **7** also involves modification of a single vinyl group, in this instance by reaction **c**. The remaining two compounds introduce a further complication. Thus, compound **6** has the same composition as compound **2** and thus must involve addition of a total of four thiocyanate moieties to the two vinyl groups. If the thiocyanate groups add in the same manner, the two structures should be identical except perhaps for the absolute stereochemistry of the addition. As stereoisomers are not detected for the other addition products, and the difference in retention times is 21.5 min, this would seem to be an unlikely explanation. An attractive explanation exists, however, for which there is good precedent in the addition reactions of HOSCN and $(SCN)_2$.[20,34−41] The thiocyanate group that quenches the cationic intermediate can form a bond to the

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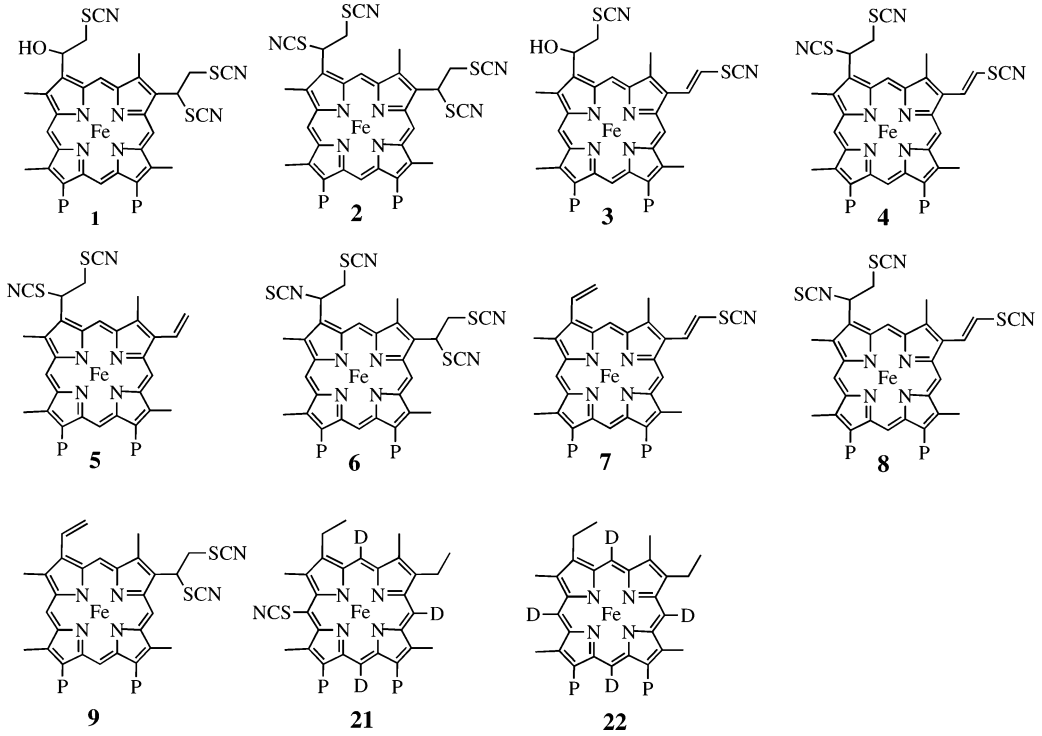
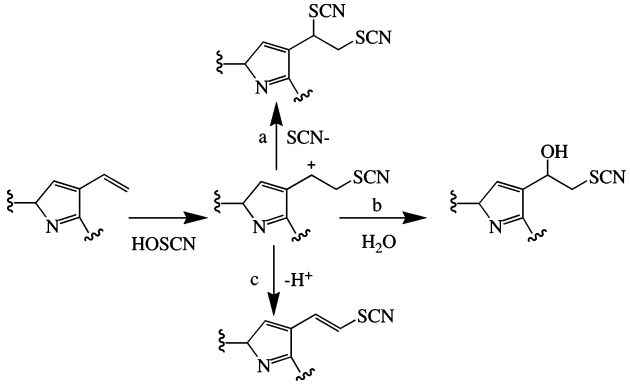
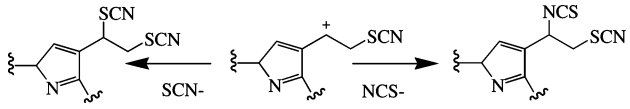


Figure 3. Structures of the products obtained in the reaction of HRP with thiocyanate and H_2O_2. The propionic acid substituents ($-CH_2CH_2CO_2H$) on the bottom two pyrrole rings of the heme are indicated by the letter P.

Scheme 1. Potential Reactions of Hypothiocyanous Acid with a Heme Vinyl Group



Scheme 2. The Two Possible Orientations of Thiocyanate Ion in Trapping of the Cationic Intermediate Yield Isomeric Products[20,34]



carbon through either the sulfur atom or the nitrogen atom, giving distinct thiocyanate or isothiocyanate products (Scheme 2). The two resulting products would be as shown for compounds **2** and **6**, although which is which remains ambiguous. The same explanation can be advanced for compound **8**, which otherwise should be identical to compound **4**. Although not unambiguous, we have assigned the thiocyanate structure to the more polar (faster eluting) product based on the argument that it should be more polar because its nitrogen is more fully exposed than it is in the isothiocyanate adduct. Chemical approaches to confirming these assignment have not been successful due to the instability of the heme adduct to thermal conditions and the difficulty in isolating pure samples of the isomers for infrared spectroscopy.[37] The alternative possibility for these two compounds, that in one of them the 2-vinyl has reacted by reaction **a** and the 4-vinyl by reaction **c**, whereas in the other the reactions occur on the opposite vinyls, is addressed below.

To approach the question of which of the reactions could occur at each of the two vinyl groups, we reconstituted *apo*-HRP with 2-cyclopropyl heme (**15**) or 4-cyclopropyl heme (**20**) (Figure 4) and incubated the reconstituted proteins with thiocyanate and H_2O_2 under the same conditions as before. The incubation of HRP reconstituted with the 2-cyclopropyl heme (**15**) yielded, in addition to unreacted prosthetic group, compounds **11**, **12**, **13**, and **14** (Figure 4). These compounds can be unambiguously assigned to the products shown in the figure by the same approach as was used to assign the products obtained with the native enzyme, but with no ambiguity concerning which vinyl group is involved in the reaction (Table 2). Compound **11** arises by addition of a thiocyanate and a hydroxyl to the 4-vinyl (Scheme 1, path **b**), compound **12** by addition of two thiocyanates (Scheme 1, path **a**), compound **13** by addition of a thiocyanate with elimination of a proton (Scheme 1, path **c**), and compound **14** addition of two thiocyanate groups, with the trapping group inverted as shown in Scheme 2. The assignment of which is **12** and which is **14** is not unambiguous but again rests on the relative polarities of the two products.

Thus, all four reactions postulated for the wild-type enzyme can occur at the 4-vinyl group. Similar incubation of HRP reconstituted with 4-cyclopropyl heme (**20**) results in the formation of compounds **16**, **17**, **18** (trace), and **19** (Figure 4). Product **16** can be attributed to addition of a thiocyanate and a hydroxyl (Scheme 1, path **b**), compound **17** to addition of two thiocyanates (Scheme 1, path **a**), compound **18**, only obtained in trace amounts, to addition of a thiocyanate and loss of a

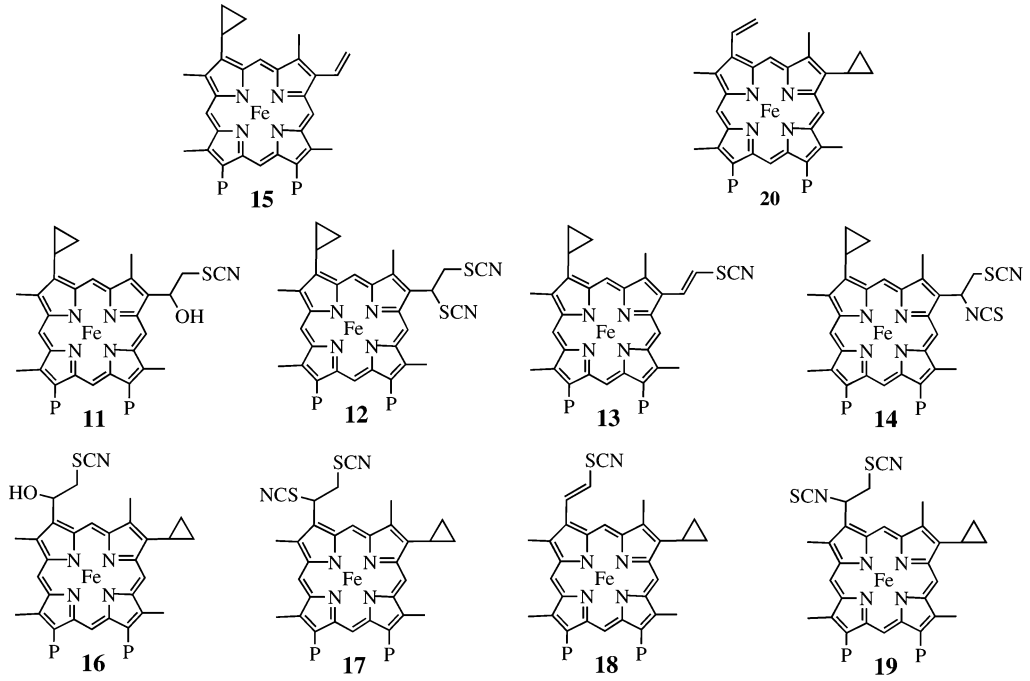


Figure 4. Cyclopropyl hemes and products from HRP reconstituted with them after reaction with thiocyanate and H_2O_2.

Table 2. Summary of the Mass Spectrometric Data on the Modified Heme Products Obtained from Incubations of 2- and 4-Cyclopropyl Heme-Reconstituted HRP with Thiocyanate and H_2O_2

compound	M^+ ion (*m*/*z*)[a]	heme[b]	SCN[c] (#)	OH (#)	H	molecular mass
11	705	630	58 (1)	17 (1)		705
12	746	630	116 (2)			746
13	687	630	58 (1)		−1	687
14	746	630	116 (2)			746
16	705	630	58 (1)	17		705
17	746	630	116 (2)			746
18	687	630	58 (1)		−1	687
19	746	630	116 (2)			746

[a] The molecular ion is usually accompanied by a strong ion 32 mass unit higher for the methanol-complexed iron porphyrin. [b] The mass of 2- and 4-cyclopropyl heme is 630. [c] The molecular mass of SCN is 58.

proton (Scheme 1, path **c**), and compound **19** to addition of two thiocyanates with the second in inverted orientation (Scheme 2). One important finding from this experiment is that addition of a thiocyanate with loss of a proton to regenerate the vinyl group occurs readily at the 4-vinyl but very poorly, if at all, at the 2-vinyl. This allows us to assign the products formed by this pathway with the native enzyme to the 4-vinyl and thus establishes the regiochemistry of the addition reactions in structures **3**, **4**, **7**, and **8**. A second significant finding is that the formation of isomeric thiocyanate and isothiocyanate trapping products (i.e., **12** and **14**, **17** and **19**) is confirmed in these simplified systems and occurs at both vinyl groups, confirming the occurrence of this process in the products formed from the native enzyme (i.e., **2** and **6**, **4** and **8**).

Reaction of HRP with Hypothiocyanous Acid HOSCN. To determine if heme modification by HOSCN occurs and to identify the products formed by this agent, we generated HOSCN by incubation of equimolar thiocyanate and H_2O_2 with LPO, which is reported to accumulate HOSCN,[25,28] followed by treatment of the solution with catalase to remove the excess H_2O_2.[30] The HOSCN thus generated was then incubated with HRP before HPLC and mass spectrometry analysis (Figure 5).

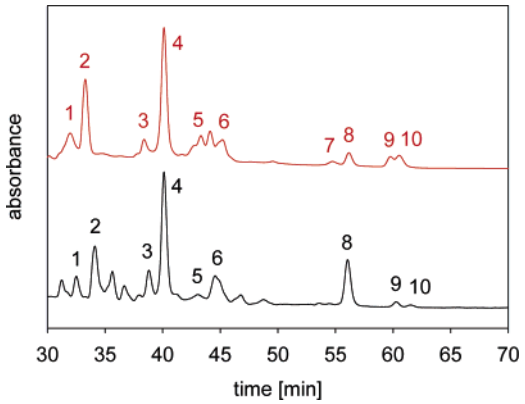


Figure 5. Differences in products distribution in: heme modified in the reaction of HRP with thiocyanogen and H_2O_2 (red line) and heme modified by HOSCN generated by LPO (black line). Absorbances of heme-derived products were recorded at 398 nm.

The product patterns were found to be very similar in the incubation with preformed HOSCN and the incubation in which the reactive species was generated in situ by reaction of HRP with thiocyanate and H_2O_2. The only significant change appears to be a modest increase in the formation of product **8**. Thus, HOSCN readily accounts for the observed prosthetic heme modification.

Model Study of the Reaction of Heme with Dithiocyanogen. A major product of thiocyanate oxidation by HRP is thought to be thiocyanogen.[25] Therefore, we generated dithiocyanogen from lead thiocyanate[34] and allowed it to react with a solution of heme (ferric protoporphyrin IX) in an organic solvent to compare the products formed in the presence and absence of the HRP protein. A comparison of the heme−thiocyanate adducts formed in the incubation of HRP with thiocyanate and H_2O_2 versus those formed in a reaction of dithiocyanogen with heme in THF solution (Figure 6) shows that the same products are formed in both cases, although there is a shift in the product distribution toward the tetrasubstituted

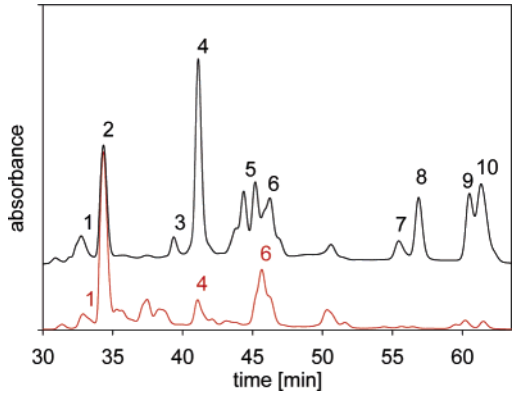


Figure 6. LC chromatogram of heme−thiocyanate adducts found in the case: HRP incubation with SCN^- and H_2O_2 (black line) and incubation of ferric heme with dithiocyanogen in THF (red line). Absorbances of heme-derived products were recorded at 398 nm.

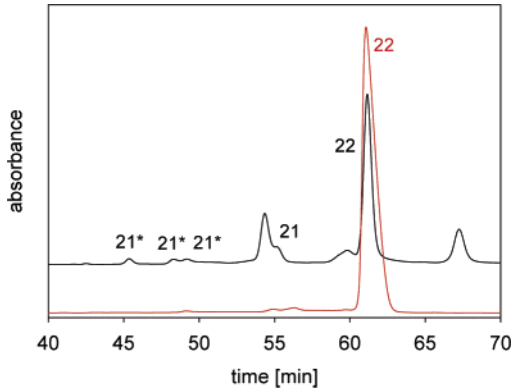


Figure 7. HPLC chromatogram of mesoheme-d_4 (red line) and UV-irradiated mesoheme-d_4 (black line). Absorbances of heme-derived products were recorded at 398 nm.

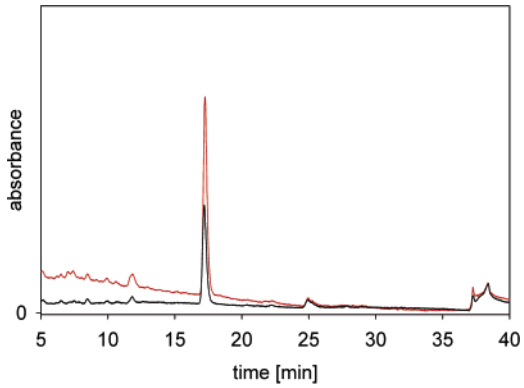


Figure 8. HPLC chromatogram of LPO before and after incubation with SCN^- and H_2O_2, followed by proteolytic digestion: native LPO digested with trypsin and protease K (red line), native LPO incubated with SCN^- and H_2O_2 before proteolytic digestion (black line). The single peak at t = 17.2 min is 1,5-dihydroxymethylheme. No modified heme products were detected.

products **2** and **6** in the model reaction because (a) the high concentration of dithiocyanogen in the model reaction diminishes products with an unreacted vinyl group, and (b) the deprotonation reaction (Scheme 1, path **c**) appears to not operate efficiently in the THF solution conditions. These results confirm that both $(SCN)_2$ and HOSCN could contribute to the observed heme modifications.

Model Study of the Reaction of Mesoheme-d_4 with the Thiocyanogen Radical. Previous studies have demonstrated that a variety of substrates, including arylhydrazines,[39] cyanide,[24] cyclopropanone hydrate,[40] acetate,[41] and azide,[20] are oxidized by HRP to radical species that react with the *δ-meso* carbon of the prosthetic heme group to give the *δ-meso*-substituted heme. Only a trace of such a product (**21**) was found in these studies when the prosthetic group of HRP was replaced with *meso*-tetradeuterated mesoheme (Figure 1), a prosthetic group without vinyl groups and therefore unable to form vinyl adducts. To determine if the thiocyanate radical, if formed, would give such adducts, we irradiated a sample of dithiocyanogen with a UV lamp,[42,43] conditions known to generate the thiocyanate radical, in the presence of mesoheme-d_4. Mesoheme was chosen for this model experiment to amplify any possible reactions with the *meso*-carbons by suppressing all reactions with the vinyl groups, as well as to simplify the product analysis. As shown in Figure 7, the reaction produces small amounts of four modified hemes with the molecular ion (m/z = 680) expected of *meso*-substitution: i.e., M^+ = 624 (mesoheme) + 58 (SCN) −2 (D). Four *meso*-substituted products are expected here because the intrinsic reactivities of the four *meso*-positions are not very different in the absence of a protein framework.

One of the four peaks (21) corresponded in HPLC retention time with the trace of a single adduct isolated from the reaction of mesoheme-d_4-reconstituted HRP with thiocyanate and H_2O_2 (Figure 1). In the enzyme, the formation of only one isomer of the adduct, presumably at the *δ-meso* carbon, is expected due to steric suppression of reaction at other *meso*-positions by the protein structure.[33,39] As many as eight such adducts could in principle be formed in the absence of the protein due to the presence of four *meso*-positions and the possibility of thiocyanate/isothiocyanate formation (21*). Three additional unassigned peaks found in chromatogram after UV irradiation of mesoheme-d_4/$(SCN)_2$ mixture (Figure 7, black line) correspond to unknown heme degradation products.

Evaluation of LPO Heme Modification During the Thiocyanate Oxidation. LPO was incubated with SCN^- and H_2O_2 under conditions similar to those used for HRP. The reaction solution was then subjected to protease digestion (trypsin + protease K) to release the covalently bound heme. The single peak observed at t = 17.2 min in the LC−MS chromatogram was identified as 1,5-dihydroxymethylheme by its retention time and mass spectrum (Figure 8), which exhibits a molecular ion at m/z 648. As 1,5-dihydroxymethylheme is the normal modified heme expected when the two covalent ester bonds to the protein are hydrolyzed, it is clear that, unlike HRP, no detectable prosthetic heme modification occurred when LPO was incubated with thiocyanate and H_2O_2. This experiment was repeated multiple times under slightly varying conditions with the same results.

Discussion

The oxidation of thiocyanate by peroxidases, including HRP and the mammalian peroxidases,[16,25,28−31] produces HOSCN and $(SCN)_2$, electrophilic species that react readily with nucleophilic sites in the macromolecules of target organisms. The present findings clearly show that formation of these reactive species is accompanied by modification of the prosthetic group of HRP

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but not of LPO, a representative mammalian peroxidase for which thiocyanate oxidation is the principal, physiologically relevant, reaction. Analysis of the nine modified prosthetic heme groups (Figure 1) found when the prosthetic group is extracted from HRP after turnover of thiocyanate establishes that all of them are generated by electrophilic attack of oxidized thiocyanate on the heme vinyl groups (Figure 3). The structures of the modified hemes are based on (a) their heme-like UV−vis spectrum, (b) their mass spectrometric molecular ions, (c) the finding that no such adducts are found when the normal heme prosthetic group is replaced by iron mesoheme, an analogue of heme in which the vinyl groups are replaced by ethyl groups (Figure 1), and (d) analysis of the regiochemistry of the reactions observed when the prosthetic heme group is replaced by either 2-cyclopropyl or 4-cyclopropyl heme, prosthetic groups in which only one of the two vinyl groups is regiospecifically retained on the heme (Figure 4). The structures of the modified hemes are secure except for an ambiguity in some instances concerning which of the two double bonds is involved in which reaction. This ambiguity exists with structures **1**, **2/6**, and **5/9**, that is, the structures that do not have a thiocyanate-substituted vinyl group. The thiocyanate vinyl group is shown by the data from the cyclopropyl heme adducts to occur preferentially at position 4 rather than position 2, making the 2-vinyl the site of other modifications when it is present. The products can all be rationalized by one of the three reactions illustrated in Scheme 1: (a) addition of thiocyanate to both carbons of a given double bond, with the internal moiety binding as either a thiocyanate (RSCN) or an isothiocyanate (RNCS) due to trapping by either the sulfur or nitrogen of NCS^- of the cationic intermediate produced by electrophilic thiocyanate addition to the terminal carbon (Scheme 2), (b) addition of thiocyanate to the terminal carbon of the vinyl group and a hydroxyl to the internal carbon, and (c) replacement of a vinyl hydrogen by a thiocyanate moiety, presumably giving the *trans*-stereochemistry of the adduct. Although ^{15}N NMR studies have shown that in the resting state of HRP SCN^- binds 6.8 Å away from the heme iron,[27] this does not help to define the regiochemistry of the additions as the position of the thiocyanate is likely to shift as the enzyme is converted to the catalytically active ferryl form.

Recently, we reported that the heme group of HRP is also modified during the oxidation of bromide to hypobromous acid (HOBr).[23] The modified prosthetic groups extracted from the HRP after incubation with bromide and H_2O_2 were produced by electrophilic addition of HOBr to one or the other, or both, of the heme vinyl groups, giving either a bromohydrin or vinyl bromide modification (i.e., Scheme 1 paths **a** and **c** if thiocyanate is replaced by Br). Interestingly, the vinyl bromide product was found in that reaction to highly favor the C-4 vinyl group, just as it does in the present situation. This difference in the relative proportions of the two products can be explained by the presence of two water molecules in the active center of HRP vicinal to the 2- but not 4-vinyl group (PDB 1h5a).[44] This difference, which places the 2-vinyl in a more aqueous, polar environment, can readily account for preferential trapping of the carbocation generated by reaction with HOSCN, $(SCN)_2$, or HOBr by either a water molecule or, in the present case, a thiocyanate anion.

Model reactions in which HRP was exposed to HOSCN preformed with LPO, or a solution of ferric heme was allowed to react with chemically generated $(SCN)_2$, demonstrated that the reactions observed during the HRP-catalyzed process were the same as those observed when the heme vinyl groups reacted with the authentic reagents. Only a small difference in product distribution was observed between the autocatalytic reaction and the reaction of HRP with preformed HOSCN (Figure 6). A somewhat larger difference in the product ratio was observed for the reaction of heme with $(SCN)_2$ in solution, as might be expected given the relatively high amount of the electrophilic reagents and the absence of a protein structure to control the reaction regiochemistry and outcome.

It is known that oxidation of substrates such as acetate,[41] azide,[20] and cyanide[24] results in attack on the sterically accessible *δ-meso* carbon of the heme prosthetic group of HRP, resulting in *δ-meso*-substitution and inactivation of the enzyme. A search for such *meso*-substitution reactions in the reaction of HRP with thiocyanate was carried out by replacing the prosthetic heme group with mesoheme-d_4, in which vinyl group reactions are not possible because these substituents are replaced by ethyl groups. Only a trace of an adduct with the molecular mass corresponding to replacement of a *meso*-deuterium by a thiocyanate was thus obtained. Nevertheless, model studies in which a solution of mesoheme-d_4 and $(SCN)_2$ was subjected to UV irradiation, conditions that generate the SCN• radical, resulted in the formation of multiple *meso*-substituted mesoheme adducts (Figure 7). Multiple adducts are expected in this experiment as all four *meso*-positions are accessible in the absence of a protein matrix. Thus, if the SCN• radical were a major product of the HRP-catalyzed oxidation of thiocyanate, significant amounts of the *meso*-substituted heme would be expected. The virtual absence of such adducts suggests that the oxidation of thiocyanate does not proceed via the radical but rather involves direct conversion of thiocyanate to HOSCN. In an earlier study of the oxidation of bromide ion, we similarly found heme modification of the vinyl groups but not *meso*-addition.[23] When we examined the much more difficult oxidation of chloride ion by HRP, we found results similar to those with bromide, except that a small but significant amount of *meso*-chlorinated prosthetic group was observed when the heme of HRP was replaced by mesoheme.[23] These results support the general inference that electrophilic reagents react with the vinyl groups but not the *meso*-positions of the heme, whereas radical reagents add to the *meso*-positions (and probably also the vinyl groups).

A key finding of this investigation is that the heme of LPO, unlike that of HRP, is not subject to modification by oxidized thiocyanate products (Figure 8). The HRP autocatalytic studies (Figure 1), and the model studies in which HRP was exposed to preformed HOSCN (Figure 5) and isolated heme to $(SCN)_2$ (Figure 6), clearly establish that the heme, both inherently and within a peroxidase protein environment, is susceptible to vinyl group modifications by these reagents. Thiocyanate is oxidized by HRP, but this reaction is not known to have any particular role in plant physiology. In contrast, the oxidation of thiocyanate is the primary physiological reaction of LPO[15] and is physiologically relevant as well for MPO and eosinophil peroxidase.[1,2,16] The present results reveal that LPO, and presumably also the other mammalian peroxidases for which thiocyanate is a critical substrate, have evolved active sites that are resistant to modification by the product formed in their catalytic turnover

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of thiocyanate. In the case of MPO, the 2-vinyl group is strongly protected against electrophilic attack by the presence of a positively charged sulfur substituent on the vinyl group. However, LPO, without this charged vinyl substituent, is still fully protected against electrophilic addition. All three of these mammalian peroxidases have one distinctive feature in common, the presence of two covalent ester bonds between the heme 1- and 5-methyls and carboxylic acid bearing protein side-chains. An attractive hypothesis is therefore that these covalent bonds sterically protect the vinyl groups, as the 2-vinyl is adjacent to the ester bond of the 1-methyl, and the 4-vinyl, although not on the same ring as the 5-methyl, nevertheless is located close to it in space. We have examined the structure of MPO, as a structure is not available for LPO. Even though the MPO structure suffers from the fact that the exceptional covalent bond to its heme vinyl group is a critical change in the relevant region of the active site, the structure suggests that the environment of the other vinyl group is sterically constricted by the ester bond to the 5-methyl group. The hypothesis that at least one role of the two ester bonds between the heme and the protein is to protect the heme vinyl groups is thus consistent with the available facts, although only a direct test of the hypothesis can unambiguously determine its validity. Experiments are currently underway with heterologously expressed site-specific mutants of LPO in efforts to obtain such evidence.

Experimental Section

Materials. HRP (EIA grade) was obtained from Roche. The 30% (w/w) H_2O_2, methanol-*d*, 2-butanone, *N*-*tert*-butyl-α-phenylnitrone, dibasic potassium phosphate, and trifluoroacetic acid were purchased from Sigma-Aldrich. Potassium hydroxide, sodium thiocyanate, citric acid, methanol, and acetonitrile were obtained from Fisher. Citrate buffer was prepared from citric acid titrated to pH 4 with 1 M KOH. Water was double distilled prior to use. 2-Cyclopropylheme and 4-cyclopropylheme were synthesized from protoporphyrin IX dimethyl ester by modification of a published procedure.[45]

Spectrophotometric measurements were performed on a Hewlett-Packard 8450A diode array spectrophotometer. The concentration of HRP was determined by using $\epsilon_{402} = 102\,000\ M^{-1}\ cm^{-1}$ and a molecular weight of 44 000.[46] The H_2O_2 concentration was standardized spectrophotometrically at 240 nm by using the molar extinction coefficient $\epsilon = 43.6\ M^{-1}\ cm^{-1}$.[47] LC−MS was performed on a Waters Micromass ZQ coupled to a Waters Alliance HPLC system (2695 separations module, Waters 2487 Dual λ Absorbance Detector) employing an Alltech C_{18} column (2.1 × 250 mm, 5 μm).

Formation of the Heme Adducts by HRP. All solutions were prepared in citrate buffer (0.05 M, pH 4). HRP 50 μL (40 μM) was incubated with NaSCN 50 μL (1.2 M) and H_2O_2 (0.4 mM, 10 equiv) at ∼25 °C for 30 min. A 50 μL aliquot of the solution was directly injected onto the LC−MS. LC−MS analysis was performed with an Alltech Alltima C_{18} column (2.1 × 250 mm, 5 μm) fitted with a guard column. Solvent A was water containing 0.1% formic acid, and solvent B was methanol containing 0.1% formic acid. The gradient program consisted of linear segments with 40% B (0−7 min), from 40% to 64% B (7−8 min), from 64% to 74% B (8−33 min), 95% B (33.1−40 min), 40% B (40.1−55 min), at a flow rate of 0.1 mL/min. The eluent was monitored at 280 and 398 nm. The settings of the mass spectrometer were as follows: capillary voltage, 3.5 kV; cone voltage, 25 V; desolvation temperature, 300 °C; source temperature, 120 °C.

Incubation of HRP with Hypothiocyanous Acid. Hypothiocyanous acid was generated by incubation of LPO (50 μL, 20 μM) with NaSCN (1 mL, 0.62 M) and H_2O_2 (0.5 mL, 1.24 M) at pH 5 in room temperature for 5 min. The reaction was carried out in water solution equilibrated to pH 5 with diluted HCl because buffer components are known to catalyze HOSCN decomposition.[30] The incubation was stopped by addition of catalase (20 μL of solution containing 1 mg of catalase in 200 μL of water) to remove excess of H_2O_2. After 2 min of incubation, the mixture was filtered with a centrifugal filter device (Amicon, 10 K MWCO). The filtrate containing the HOSCN (1 mL) was added to 1 mL of 100 μM HRP dissolved in water at pH 5. The sample was incubated for 30 min at room temperature and was then analyzed by LC−MS using the standard gradient given above for thiocyanate−heme adducts.

Formation of the Heme Adducts with Dithiocyanogen. Dithiocyanogen was generated by addition of 20 μL of bromine to 0.13 g of $Pb(SCN)_2$ in 4 mL of dry CH_2Cl_2. The mixture was stirred at room temperature for 1 h (as long as the solution became colorless), filtered, and kept in the dark after addition of a radical inhibitor (5 mg of *N*-*tert*-butyl-α-phenylnitrone). The concentration of dithiocyanogen generated in this manner was estimated as 86.7 mM ($\epsilon = 147\ M^{-1}\ cm^{-1}$ at 297 nm in CCl_4).[48] A 10 mg sample of hemin was dissolved in 20 mL of freshly distilled dry THF. A trace of insoluble hemin was filtered off. To this was quickly added 3.5 mL of a dithiocyanogen solution in CH_2Cl_2, and the mixture was stirred for 30 min at room temperature in the dark in the presence of 5 mg of the radical inhibitor *N*-*tert*-butyl-α-phenylnitrone. The solvent was evaporated under reduced pressure. The crude product was extracted with acetone and again dried under vacuum, giving 10.4 mg of red solid. This residue was dissolved in methanol and analyzed by LC−MS using the conditions that were used for the analysis of modified hemes in the HRP/SCN^-/H_2O_2 system.

UV Irradiation of Deuterated Mesoheme in the Presence of Dithiocyanogen: LC−MS Spectrometry. The dithiocyanogen (50 μL, 2 mM) generated according to the above method (without the radical inhibitor) was added to the 50 μL of 20 μM deuterated mesoheme in THF. The mixture was placed in a 100 μL quartz cuvette and irradiated for 1 h at 190 nm using UV lamp. The reaction mixture was then analyzed by LC−MS under the same conditions as were used for determination of modified hemes in the HRP/SCN^-/H_2O_2 system.

Preparation of Mesoheme-d_4. Deuterium substitution at the four methine carbons was accomplished as reported previously with small modifications.[23,49] A mixture of magnesium (160 mg), iodine (300 mg), and anhydrous diethyl ether (5 mL) was refluxed under N_2 until colorless before being filtered. The filtrate was evaporated to dryness under vacuum, and the residue was dissolved in anhydrous pyridine (5 mL) and methanol-*d* (3 mL). The resulting solution was added to a solution of mesoporphyrin IX dimethyl ester (20 mg) in 1 mL of dry pyridine, and the combined solution was heated overnight at reflux under an atmosphere of N_2. Deuteration of iron mesoporphyrin IX dimethyl ester gives a better deuteration yield than deuteration of mesoporphyrin IX dimethylester followed by iron insertion, probably because back exchange of the deuterium in the iron insertion step is avoided. The cooled reaction mixture was diluted with diethyl ether (40 mL), extracted with 1 M HCl (2 × 50 mL) and water (50 mL), dried over Na_2SO_4, and finally evaporated to dryness under reduced pressure. The final residue was dissolved in 30 mL of radical-free THF, and 40 mL of 2 M NaOH was added. The reaction was carried out at room temperature overnight. The THF was evaporated, and the solution was acidified to pH = 7 (1 M HCl) and extracted with CH_2Cl_2 (2 × 30 mL). The organic phase was washed with water (2 × 30 mL), dried over Na_2SO_4, and concentrated to dryness under vacuum, giving 17 mg of pure deuterated mesoheme. LC−MS showed the presence of a single tetradeuterated mesoheme peak with *m*/*z* (intensity %) 624 (100).

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(49) Kenner, G. W.; Smith, K. M.; Sutton, M. J. *Tetrahedron Lett.* **1973**, *14*, 1303−1306.

Removal of Heme from HRP. *apo*-HRP was prepared according to a previously described method.[41] HRP (10 mg) was dissolved in 1 mL of 25 mM phosphate buffer (pH 7.0), and into this 100 μL of 3 M HCl was added to bring the pH to 1.3. The HRP solution was extracted with 2-butanone (2 mL $\times$ 2) to remove the heme, and then immediately neutralized with 1 M NaOH. The apoprotein in the aqueous phase was passed through a PD-10 column eluted with 25 mM phosphate buffer (pH = 7.0). Fractions containing *apo*-HRP were combined and concentrated with an ultracentrifugal filter device (10K MWCO, Millipore Amicon), affording $\sim$0.4 mL of *apo*-HRP with $AU_{278} = 0.55$ after 8-fold dilution, $R_z = 0.03$ (R_z = Reinheit Zahl). The residual heme in the *apo*-HRP was less than 1%.

Reconstitution of HRP with 2- and 4-Cyclopropylheme. *apo*-HRP (50 μL, $AU_{278} = 0.4$ after 10-fold dilution) was added to 450 μL of phosphate buffer (25 mM, pH 7.0). To this solution was added 2-cyclopropylheme in DMSO (15.8 μL, 2 equiv, $AU_{396} = 0.811$ after 100-fold dilution). After standing for 2 h, the solution was passed in phosphate buffer (25 mM, pH 7.0) through a DEAE (DE52) column equilibrated in the same buffer and was concentrated with a centrifugal filter device (Amicon, 10K MWCO): $AU_{400} = 1.07$ after 2-fold dilution, $R_z = 1.6$.

apo-HRP (100 μL, $AU_{278} = 0.4$ after 10-fold dilution) was added to 900 μL of phosphate buffer. To this was added 4-cyclopropylheme in DMSO (4 μL, 2 equiv, $AU_{394} = 0.729$ after 1000-fold dilution). The same procedure was then followed as in the case of 2-cyclopropylheme. The final solution had $AU_{398} = 0.38$ after 10-fold dilution, $R_z = 2.5$.

To *apo*-HRP (800 μL, $AU_{278} = 0.55$) in phosphate buffer pH = 7 was added a 7 μL ($\sim$2 equiv) solution of deuterated mesoheme in methanol ($AU_{390} = 1.05$ after 500-fold dilution). The same procedure was then followed as above. The final solution had $AU_{398} = 0.77$ after 10-fold dilution, $R_z = 2.6$. The reconstituted HRP proteins were tested for activity as described previously before being treated with SCN^- under the same conditions.[41]

Oxidation of Thiocyanogen by LPO. LPO (40 μM, 200 μL) in citrate buffer was mixed with 100 μL of 0.5 M citrate buffer at pH 4.4 and 150 μL of 2 M SCN^- aqueous solutions. To this was added 50 μL of H_2O_2 (4 mM final, 25 equiv) in citrate buffer. The resulting solution was incubated at room temperature for 50 min. As a control experiment, LPO was also incubated with 25 equiv of H_2O_2 in the absence of SCN^-. The reaction solutions were then concentrated with an ultracentrifugal filter device (10 000 MWCO, Millipore Amicon) and diluted with water. This step was repeated twice more. The final solution was dissolved in bis-tris propane buffer (0.1 M, pH 8.2) to give a 130 μL final volume. The solution was then heated at 90 °C for 5 min to denature the protein before adding $CaCl_2$ (15 μL, 10 mM) to give a final $CaCl_2$ concentration of 1 mM. After 15 min, trypsin (20 μg in 30 μL of suspension buffer, Promega Inc.) was added, and the solution was incubated at 37 °C overnight. $CaCl_2$ (45 μL, 10 mM) was then added to the solution to give a final $CaCl_2$ concentration of $\sim$3 mM. Protease K (10 μL, 10 mg/mL) was added, and the solution was incubated at 37 °C overnight. A 50 μL aliquot of the solution was directly injected onto the LC−MS system following a previously described protocol.[23] An 80 μL aliquot of the solution was injected into an HPLC equipped with an Alltech Altima C18 column (150 $\times$ 4.6 mm) and a guard column. The column was eluted with a gradient program consisting of linear segments (solvent A, water (0.1% TFA), solvent B, acetonitrile (0.1% TFA)) as follows: 25% B (0−2 min), from 25% to 35% B (2−22 min), from 35% to 48% B (22−35 min), from 48% to 95% B (35−36 min), and 95% B (36−40 min).

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Supporting Information Available: MS spectra of compounds **1**−**22**. This material is available free of charge via the Internet at http://pubs.acs.org.

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