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Formation of Cysteine Sulfenic Acid by Oxygen Atom Transfer from Nitrite

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Abstract: Cysteine sulfenic acid CysS(O)H is shown to be formed for the reaction of cysteine (CysSH) with aqueous nitrite and the water-soluble ferriheme models Fe^{III}(TPPS) (TPPS = *meso*-tetra(4-sulfonatophenyl)porphyrinato) or Fe^{III}(TMPS) (TMPS = *meso*-tetra(sulfonatomesityl)porphyrinato) at pH 5.8 and 7.4. The other product is the respective ferrous nitrosyl complex Fe^{II}(Por)(NO) (Por = TPPS or TMPS). Analogous oxygen atom transfers (OAT) were seen when glutathione (GSH) was used as the substrate. The sulfenic acids, CysS(O)H and GS(O)H, are transient species since they react rapidly with excess thiol to give the respective disulfides, so their presence as reactive intermediates was demonstrated by trapping with dimedone and detecting the resulting adduct using LC/MS. Preliminary kinetics studies are consistent with rate-limiting OAT from a ferric nitro complex Fe^{III}(Por)(NO_2^-) to CysSH, although this reaction is complicated by a competing dead-end equilibrium to form the thiolate complex (Fe^{III}(TPPS)($CysS^-$).

Modifications in protein cysteine (CysSH) are increasingly documented as signals to oxidative stress and may be important factors in a variety of diseases.[1] The most common modification is oxidation to disulfides. However, another oxidation product is the somewhat elusive cysteine sulfenic acid (CysS(O)H), which has drawn increasing interest as a potentially key post-translational modification of proteins in redox regulation.[1d,2] Sulfenic acids are formed by reactions of protein thiols with hydrogen peroxide, alkyl hydroperoxides, or peroxynitrite[1b] and are readily transformed by excess thiols into the respective disulfides or undergo further oxidation to sulfinic and sulfonic acids. The potential importance of sulfenic acid modifications has led to new tools designed to detect these reactive species.[3]

In this context we report that sulfenic acids are formed by ferriheme-mediated oxygen atom transfer (OAT) to cysteine (CysSH) and glutathione (GSH) from nitrite ion at physiologically relevant pH values (for example, eq 1, where Fe^{III}(TPPS) (**1**) is a water-soluble heme model complex).[4]

$$\underset{(\mathbf{1})}{Fe^{III}(TPPS)} + NO_2^- + CysSH \rightarrow \underset{(\mathbf{2})}{Fe^{II}(TPPS)(NO)} + CysS(O)H \quad (1)$$

In an earlier report, we demonstrated the analogous OAT from aqueous solution containing **1** and $NaNO_2$ to a water-soluble, sulfonated phosphine and to dimethyl sulfide (DMS) to give the respective phosphine oxide and sulfoxide (DMSO) products in addition to the ferrous nitrosyl Fe^{II}(TPPS)(NO) (**2**).[5] Analogous OAT has been seen by Kurtikyan et al. in the reaction of DMS with Fe^{III}(TPP)(NO_2)[4] in sublimed layers giving DMSO plus Fe^{II}(TPP)(NO).[6] In contrast, the reaction of aqueous CysSH and GSH with **1** plus $NaNO_2$ gave the disulfides cystine and GSSG as the respective products.[5] We proposed that these disulfides were the likely results of trapping the OAT generated sulfenic acid intermediates (RS(O)H, R = G or Cys) by excess thiol (eq 2),[7] but there was no direct evidence to support this hypothesis.[5] Here, we use another trapping agent dimedone to demonstrate that the proposed sulfenic acid intermediates are indeed formed.

$$RS(O)H + RSH \rightarrow RSSR + H_2O \quad (2)$$

Figure 1 illustrates the optical spectral changes that occurred when CysSH (final concentration 0.5 mM) was added to a solution of **1** (8.6 μM) and $NaNO_2$ (10 mM) in deaerated pH 5.81 phosphate buffer (10 mM). The initial spectrum displays a Soret band at 393 nm and a Q-band at 528 nm, characteristic of **1**, that do not change significantly when NO_2^- at this concentration was added. This insensitivity is attributed to the low equilibrium constant for forming Fe^{III}(TPPS)(NO_2^-) under these conditions ($K_{nitrite}$ = 3 M^{-1}).[8] However, when cysteine (0.5 mM) was added to this solution, there were immediate red shifts in both bands, followed by spectral changes over the period of 10−12 min that indicated the formation of the ferrous nitrosyl complex **2** (λ_{max} = 413 nm, 543 nm). When the analogous reaction was carried out using glutathione as the substrate, similar spectral changes were seen over this time frame (see Supporting Information Figure S-1).

Although CysSH is known to reduce ferric complexes and ferrihemes,[9] there was no significant reduction of **1** in the absence of NO_2^- over the time frame described by Figure 1, although such reduction did occur over several days (Figure S-2). In aerated solution there was complete decomposition of the heme as evidenced by bleaching of the Soret and Q-bands (data not shown).

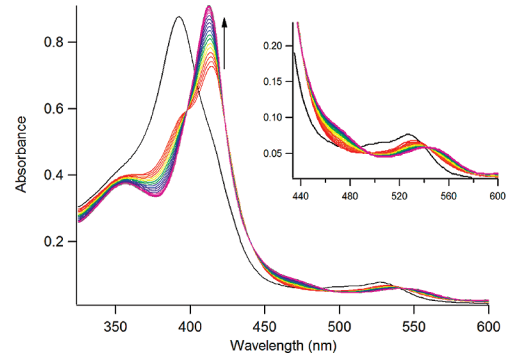

Figure 1. Optical spectral changes upon adding cysteine (0.5 mM) to a buffered aqueous solution of Fe^{III}(TPPS) (8.6 μM) and $NaNO_2$ (10 mM). Addition of CysSH leads to an immediate red shift of the Soret and Q-bands in the initial spectrum of Fe^{III}(TPPS) (black) followed by a slower absorbance increase at 413 nm (red to purple) indicating the formation of Fe^{II}(TPPS)(NO). The inset shows the magnified Q-band region.

The initial spectral shifts suggest a direct interaction of the RSH ligands with the ferric porphyrin. In this context, addition of CysSH (0 to 3 mM) to a pH 5.81 buffered solution of **1** not containing nitrite leads to a shift in the Soret band from 393 to 417 nm (Figure 2) with an isosbestic point at 408 nm indicating a single binding

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event. The latter Soret band λ_{max} is consistent with the formation of a low spin heme Fe^{III} ligated to a single thiolate, that is, $Fe^{III}(TPPS)(RS^-)$ (eq 3).[10] The X-band EPR spectrum of the solution at 110 K is also consistent with this conclusion, showing the formation of a new $S = {}^1/_2$ species ($g \approx 2$) consistent with a low spin $Fe^{III}(TPPS)(RS^-)$, but gave no indication of the high spin $Fe^{III}(TPPS)$ complex ($g \approx 6$)[11] (Figure S-3).

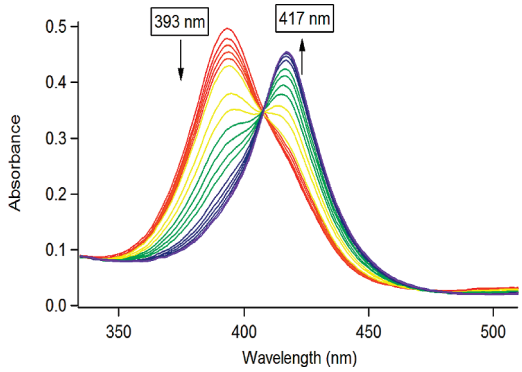


Figure 2. Spectral changes observed as cysteine (0−3 mM) was titrated into a solution of $Fe^{III}(TPPS)$ (5 μM) at pH 5.81.

$$Fe^{III}(TPPS) + CysSH \rightleftharpoons Fe^{III}(TPPS)(CysS^-) + H^+ \qquad (3)$$

A Job plot (Figure S-4) and a Hill analysis (not shown) each indicated the binding stoichiometry to be 1:1. The spectral changes in Figure 2 give an apparent binding constant K_{app} of $(2.80 \pm 0.06) \times 10^3$ M^{-1} for eq 3 at pH 5.81. K_{app} values were similarly measured at seven different pH values ranging from 4.5 to 6.25. According to the derivation described in Supporting Information Scheme S-1, a plot of $K_{app}(1 + K_{Fe}[H^+]^{-1})$ vs $[H^+]^{-1}$ (where K_{Fe} is the known acid dissociation constant for $Fe^{III}(TPPS)$, $10^{-7.0}$ M)[12] should be linear with an intercept K_1, which is the binding constant of CysSH to Fe(TPPS). The slope of this plot (Figure 3) is the product K_1K_{1a}, where K_{1a} is the acid dissociation constant of the coordinated CysSH. From the data plotted in Figure 3 the binding constant for the protonated thiol was calculated to be 230 ± 50 M^{-1} and the pK_a of the coordinated cysteine to be 4.7 ± 0.1. The latter value is considerably lower than the pK_a of free cysteine (8.3)[13] as would be expected for CysSH coordination to Fe(III).

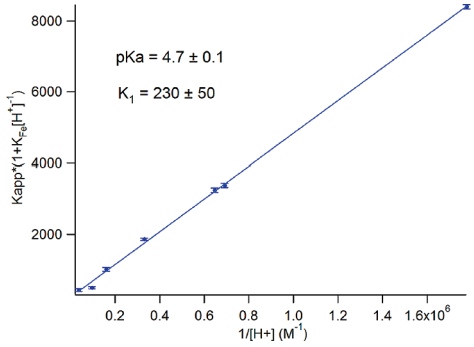


Figure 3. The pK_a of the coordinated CysSH was determined to be 4.7 ± 0.1 from the plot of $K_{app}(1 + K_{Fe}[H^+]^{-1})$ versus $[H^+]^{-1}$ over the pH range 4.50 to 6.25. The intercept is the binding constant of the protonated thiol CysSH to $Fe^{III}(TPPS)$ ($K_1 = 230 \pm 50$ M^{-1}), while the slope/intercept ratio is the acid dissociation constant K_{1a} of the coordinated CysSH.

We have carried out some preliminary kinetics studies of the reaction shown in eq 1. As seen in Figure 4, the temporal optical absorbance changes are not exponential and, indeed, appear to be nearly zero order in the early stages. However, it is worth noting that, at pH 5.81, the initial rates calculated proved to be linearly dependent on $[NO_2^-]$

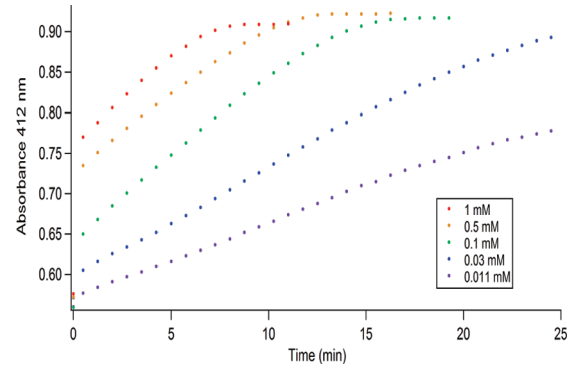
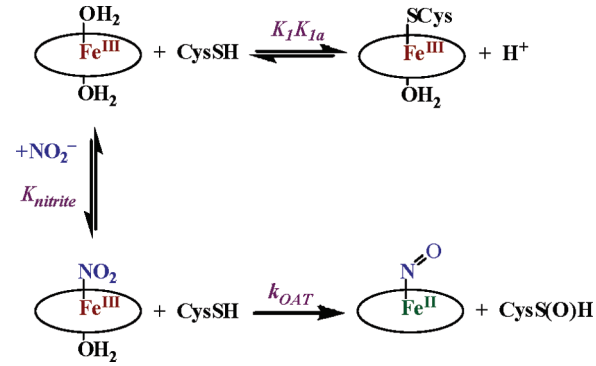


Figure 4. Kinetics traces for the formation of $Fe^{II}(TPPS)(NO)$ at 412 nm from $Fe^{III}(TPPS)$ (9.5 μM) and nitrite (10 mM) at different cysteine concentrations (0.011 to 1.0 mM). The time needed to achieve the final spectrum increases with decreasing [CysSH].

between 1.0 and 10 mM (Figure S-5) and on [**1**] but nearly independent of [CysSH] between 0.1 and 1.0 mM (Figure 4). This behavior can be explained by a mechanism where $Fe^{III}(TPPS)(CysS^-)$ itself is inactive in the OAT chemistry, so that eq 3 constitutes a dead-end equilibrium. Instead, oxygen transfer to CysSH occurs from the ferric nitrite complex $Fe^{III}(TPPS)(NO_2^-)$ (Scheme 1). Although we do not directly observe the latter species under the reaction conditions, by analogy to the observations of Kurtikyan et al.,[6] we infer that this is the N-coordinated nitro complex.

Scheme 1. Proposed Pathways for OAT from $Fe^{III}(NO_2^-)$ to Cysteine



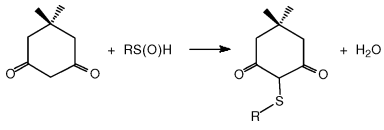
If eq 3 were a dead-end equilibrium as shown in Scheme 1, the rate of formation of **2** would be

$$\frac{d[\mathbf{2}]}{dt} = \frac{k_{OAT}K_{nitrite}[Fe^{III}_{total}][NO_2^-][CysSH]}{(1 + K_{nitrite}[NO_2^-] + K_1K_{1a}[CysSH][H^+]^{-1})} \qquad (4)$$

where $[Fe^{III}_{total}] = [\mathbf{1}] + [Fe^{III}(TPPS)(NO_2)] + [Fe^{III}(TPPS)(CysS)]$ (see Supporting Information Scheme S-2 for the full derivation). Under the reaction conditions the product $K_{nitrite}[NO_2^-]$ is $\ll 1$, so the rate should be first order in $[NO_2^-]$. However, this is not the case for $K_1K_{1a}[CysSH][H^+]^{-1}$, which, for example, equals ~0.58 at 0.2 mM CysSH and pH 5.81. Thus, in this concentration region, the effect of increasing [CysSH] on the denominator largely cancels the effect of increasing [CysSH] on the numerator of eq 4. On the other hand, as predicted by eq 3, the reaction rate becomes much more sensitive to [CysSH] at concentrations below 0.1 mM for this pH and approaches first order behavior at the lower concentrations (data not shown).

As noted above, we have suggested[5] that the initial products of OAT to the thiols CysSH and GSH are the respective sulfenic acids RS(O)H, which subsequently are intercepted by excess thiol to give the respective disulfides (eq 2). To test this hypothesis, the OAT reactions

were carried out in the presence of dimedone, which has been shown to intercept sulfenic acids to give dimedone thioethers (eq 5).[3,14]



(5)

When cysteine (1 mM) or glutathione (1 mM) were added to a pH 5.81 solution of **1** (8.6 μM) also containing $NaNO_2$ (10 mM) and dimedone (1.0 mM),[15] the reactions proceeded as before to form **2** with no discernible differences in the rates or in the spectrally intense products. After complete conversion to **2**, the product solutions were analyzed using LC/MS as described in the Supporting Information. For CysSH as substrate, the cysteine dimedone thioether eluted at 7.5 min using a Vydac, C-18 MS column or 14.9 min when using a Vydac small pore column as confirmed by the *m*/*z* peak at 260.09 (Figure S-6). When GSH was the substrate, the glutathione dimedone thioether eluted at 12 min using a Vydac, C-18 MS column or 15 min using a Vydac small pore column as confirmed by the *m*/*z* at 446.15 (Figure S-7). Thus, in both cases, sulfenic acid intermediates are generated by this reaction system. No dimedone thioethers were observed in the absence of the ferriheme.

The relatively low pH of these studies was defined by the tendency of **1** to undergo hydrolysis and form oxo-bridged dimers at higher pH. While such media are relevant to the chemistry of ischemic tissue, it was considered important to examine the generality of such OAT at pH 7.4. For this reason, the analogous reactions were probed both at pH 5.81 and at pH 7.40 using the ferriheme model Fe^{III}(TMPS) (**3**),[4] which does not display such dimer formation. At pH 7.40 (10 mM phosphate buffer), the apparent binding constant of nitrite to **3** was determined to be 1.2 M^{-1}. Such coordination at very high NO_2^- concentrations led to spectral shifts of the Soret and Q-bands to 419 and 541 nm consistent with formation of a low spin Fe^{III} porphyrinato complex (data not shown). In analogy to the reactions of **1** described above, addition of CysSH (1 mM) to a solution of **3** (8.6 μM) and nitrite (10 mM) at either pH 5.81 or pH 7.40 resulted in immediate spectral changes consistent with formation of a cysteinato complex as in eq 3. This was followed by slower spectral changes indicating the formation of Fe^{II}(TMPS)(NO) (**4**) (eq 6).

$$\underset{(\mathbf{3})}{Fe^{III}(TMPS)} + NO_2^- + RSH \rightarrow \underset{(\mathbf{4})}{Fe^{II}(TMPS)(NO)} + RS(O)H \quad (6)$$

These observations are fully consistent with the reaction pattern described by Scheme 1 whereby OAT to the substrate occurs from the ferric nitro complex $Fe^{III}(TMPS)(NO_2^-)$. One additional observation is that the formation of the ferrous nitrosyl **4** proceeds more slowly at pH 7.40 than at 5.81. This behavior is likely due to the pH dependence of eq 3 as well as the increased importance of another dead-end equilibrium involving the hydroxo complex (eq 7, pK_a 6.9).[16]

$$Fe^{III}(TMPS)(H_2O)_2^{3-} \rightleftharpoons Fe^{III}(TMPS)(H_2O)(OH)^{4-} + H^+ \quad (7)$$

When the analogous experiments with **3** were carried out in the presence of added dimedone as a trapping agent (1 mM), the respective dimedone thioethers were seen by LC/MS for both CysSH and GSH at pH 5.81 and at pH 7.40 (Figure S-8). Thus, we conclude that the sulfenic acid intermediates are formed at both pH values. Notably, increasing the concentration of CysSH or GSH led to a decrease in dimedone thioether formation while increasing the dimedone concentration led to a marked increase in the amount of thioether detected (Figure 5). Both of these observations are consistent with the literature

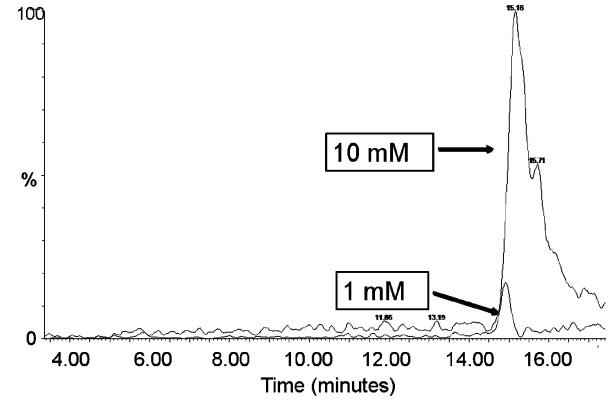


Figure 5. LC-MS traces for the analysis of cysteine sulfenic acid formed in the reaction of CysSH with **3** and NO_2^- at pH 7.4. More dimedone thioether is generated when higher concentrations of dimedone trapping agent are used. The area of the dimedone-thioether peak eluting on a Vydac small pore column increases from 7 to 43, as the concentration of dimedone increases 10-fold.

reports that competitive trapping of the sulfenic acids by the thiol substrate (eq 2) is faster than trapping by dimedone (eq 6).[17]

Neither of the dimedone thioethers were observed by LC/MS when control reactions were carried out at pH 7.40 in the absence of **3** under otherwise identical conditions. Thus we conclude that over the time frame of these experiments, relevant concentrations of GS(O)H or CysS(O)H are only formed if the iron is present to mediate their formation by OAT.

In summary, we have demonstrated that the combination of nitrite and the ferri-heme model Fe^{III}(TPPS) (at physiologically relevant pH values) leads to facile oxygen atom transfer to the biologically important thiols cysteine and glutathione to generate the respective sulfenic acids. This unique reactivity extends the known chemistry leading to formation of these highly reactive thiol oxidation products. Understanding the potential mechanisms for forming these transient intermediates will help elucidate their importance in thiol mediated redox regulation. In this context ongoing kinetics studies of the reactions illustrated by eqs 1 and 6 will provide insight into these mechanisms. We are also investigating whether such OAT pathways extend to protein thiols.

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Supporting Information Available: Description of experimental procedures and derivations of pK_a plots and of eq 4 plus figures showing spectral changes for OAT to GSH, EPR experiment, Job plot binding stoichiometry, nitrite effect on OAT kinetics, and LC-MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(4) TPPS = tetra(sulfonatophenyl)porphyrinato; Fe^{III}(TPPS) = $Fe^{III}(TPPS)(H_2O)_2^{3-}$ (Na^+ salt). TPP = *meso*-tetraphenylporphyrinato. TMPS = *meso*-tetra(sulfonatomesityl)porphyrinato; Fe^{III}(TMPS) = $Fe^{III}(TMPS)(H_2O)_2^{3-}$ (Na^+ salt).

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