Novel Application of 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole To Identify Cysteine Sulfenic Acid in the AhpC Component of Alkyl Hydroperoxide Reductase†

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Received September 4, 1997⊗

ABSTRACT: The trapping of a sulfenic acid within the fully active C165S mutant of the AhpC peroxidase protein from *Salmonella typhimurium* was investigated. The electrophilic reagent employed in these studies, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), has previously been used to modify thiol, amino, and tyrosine hydroxyl groups in proteins; at neutral pH only cysteinyl residues of AhpC proteins are modified. The peroxide-oxidized C165S mutant of AhpC incubated with NBD-Cl gave a product with an absorbance maximum at 347 nm, whereas the thiol-NBD conjugate formed from the reduced protein absorbed maximally at 420 nm. Electrospray ionization mass spectrometry of the modified proteins allowed identification of the species absorbing at 347 nm as a Cys-S(O)-NBD derivative containing one additional oxygen relative to the Cys-S-NBD product. The C165S conjugates with Cys-S(O)-NBD and Cys-S-NBD had no peroxidase activity when compared to unreacted C165S and wild-type AhpC, but were both reactivated through removal of NBD by DTT. Oxidized C165S was also modified by dimedone, a common sulfenic acid reagent, to give the expected inactivated conjugate of higher mass. This reagent was not removed by DTT and blocked any further reaction of the protein with NBD-Cl. NBD modification of *Enterococcus faecalis* NADH peroxidase, a well-characterized flavoprotein with an active-site sulfenic acid (Cys-SOH), also yielded the spectrally-distinguishable NBD conjugates following incubation of NBD-Cl with oxidized and reduced forms of the denatured peroxidase, indicating a general utility for this reagent with other sulfenic acid-containing proteins. A significant advantage of NBD-Cl over previously-used sulfenic acid reagents such as dimedone is in the retention of the sulfenic acid oxygen in the modified product; differentiation between protein-associated thiols and sulfenic acids is therefore now possible by means of both visible absorbance properties and mass analyses of the NBD-modified proteins.

The alkyl hydroperoxide reductase system from *Salmonella typhimurium,* comprised of AhpF and AhpC*,* protects the cell during aerobic metabolism from the toxic and mutagenic effects of reactive oxygen species (*1*−*3*). AhpF is a thioredoxin reductase-like pyridine nucleotide-dependent flavoprotein that is directly involved in the reduction of AhpC through redox-active cystine disulfide centers (*4*−*6*). AhpC contains two redox-active intersubunit disulfide bonds per dimer that were shown to exist in an antiparallel arrangement (*7*). We have previously presented strong evidence demonstrating that AhpC, like the well-characterized NADH peroxidase, catalyzes the cysteine-mediated reduction of peroxide substrates with the concomitant formation of a cysteinesulfenic acid (Cys-SOH) within the enzyme (*7*). AhpC differs from NADH peroxidase in that a second cysteine lies in close proximity to the nascent cysteine sulfenic acid, leading to rapid condensation of the two to regenerate the active-site disulfide bond of the oxidized wild-type enzyme.

Our previous studies focused on single (C46S, C165S) and double (C46,165S) AhpC mutants to test the importance of each of these cysteine residues in catalysis (*7*). We showed that only the C165S has full peroxidatic activity compared with wild-type AhpC, and that the peroxidase activity of C165S requires an excess of AhpF. Oxidation of C165S by H_2O_2 to generate the cysteine sulfenic acid-containing protein (Cys46-SOH) was confirmed by (i) the ability of this form of the enzyme to be reduced by 1 equiv of NADH in the presence of AhpF, (ii) the reactivity of this species toward TNB[1] to generate a mixed disulfide, and (iii) the sensitivity of this species to further oxidation by air or excess H_2O_2. These tests also demonstrated that C46S is not similarly oxidized by H_2O_2, although cysteine residues within both C165S and C46S are highly accessible to the thiol reagent DTNB. These results confirmed the role of Cys46 as the residue responsible for the reduction of alkyl hydroperoxides; Cys165 is important in regenerating the disulfide bond to prevent further oxidation of the sulfenic acid.

Cysteine sulfenic acids have been proposed as intermediates in a number of catalytic and regulatory proteins (*8*). In addition, this species has been identified definitively by cryocrystallography and ^{13}C-NMR techniques in the active site of the NADH peroxidase protein from *Enterococcus faecalis* (*9, 10*). Difficulties in identifying cysteine sulfenic acids arise as a result of their instability due to self-dimerization to form thiosulfinates, reactivity toward thiols to form disulfides, and reactivity toward mild oxidants to generate sulfinic or sulfonic acids (*11*). To definitively

† This research was supported by NIH Grant GM-50389 and Council for Tobacco Research Awards SA006 and 4501 to L.B.P.

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1997.

[1] Abbreviations: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; TNB, 2-nitro-5-thiobenzoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ESIMS, electrospray ionization mass spectrometry; DTT, 1,4-dithiothreitol; dimedone, 5,5-dimethyl-1,3-cyclohexanedione.

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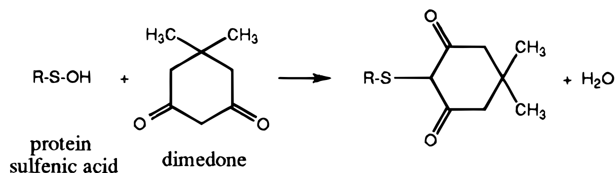
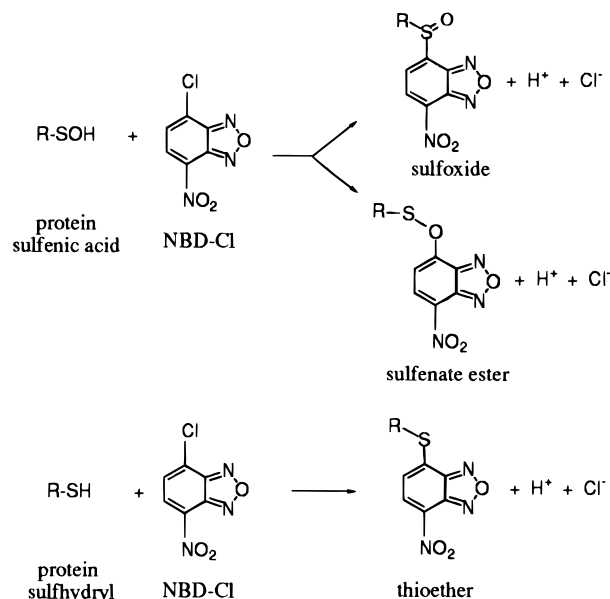
Scheme 1



Scheme 2



demonstrate the formation of a Cys-SOH in the C165S mutant of AhpC, we sought a reagent that, once reacted, would retain the oxygen in the final product. A reagent commonly used to test for the presence of a sulfenic acid, dimedone, is a nucleophilic reagent that attacks the sulfenate sulfur, displacing hydroxide and forming a thiol adduct (Scheme 1; *12, 13*). This reagent is not spectroscopically detectable; reaction with the protein sulfenic acid can only be inferred from incorporation of the radiolabeled compound, inhibition of activity, and/or mass spectrometric analyses (*12, 14*). In these studies we chose the electrophilic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) because it had several potential advantages over the nucleophilic reagents previously used. Earlier results had shown that, upon reaction with a thiol, NBD-Cl gives a characteristic absorbance at 420 nm (*15*). Although this reagent can also react with tyrosyl and amino groups, particularly at basic pH, the spectral and fluorescence properties of these products differ from those of the thiol adduct (λ_{max} = 382 and 480 nm for tyrosyl- and amino-NBD adducts, respectively; *16*–*18*). Given its electrophilic nature, we predicted that NBD-Cl reacted with a Cys-SOH should yield either a sulfoxide or sulfenate ester product depending on whether the sulfur or the oxygen of the Cys-SOH acts as the nucleophilic center (Scheme 2). Our expectations were that this reagent would be useful as a spectral probe and that the product would be detectable by electrospray ionization mass spectometry (ESIMS) with a difference of 16 amu between the thiol adduct, Cys-S-NBD, and the sulfenic acid adduct, Cys-S(O)-NBD.[2]

We describe herein the trapping and identification, by both visible spectroscopy and mass spectrometry, of the cysteine

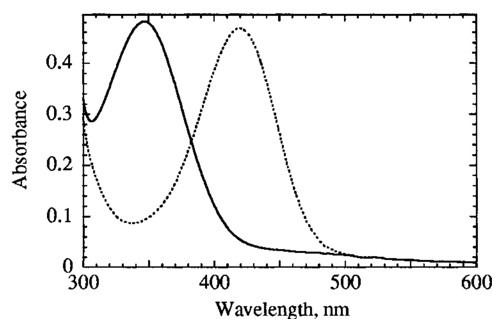


FIGURE 1: Spectroscopic characteristics of NBD-modified AhpC mutants. The Cys-S(O)-NBD conjugate was generated by incubating 30 nmol of C165S AhpC with 1 equiv of H_2O_2 under anaerobic conditions for 15 min, followed by incubation with 2 equiv of NBD-Cl (0.6 mL total volume) for 30 min in the standard buffer containing 50 mM KCl (solid line). Modified proteins were concentrated and rediluted into standard buffer three times in a Centricon CM-30 prior to absorbance measurements. The Cys-S-NBD conjugate was generated under conditions similar to the Cys-S(O)-NBD conjugate, but without pretreatment with H_2O_2 (dotted line). The rate of formation of the Cys-S(O)-NBD conjugate was a little slower than that of the Cys-S-NBD conjugate, with spectral changes half complete by about 5 and 2$^1/_2$ min, respectively.

sulfenic acid intermediate of the H_2O_2-oxidized C165S mutant of AhpC. Modification of the protein at the single Cys46 residue present in the active site is shown by thiol and activity assays, spectroscopic properties, and reactivation of the modified protein with DTT. A more general utility for this reagent, NBD-Cl, as a trapping agent for cysteine sulfenic acids has been demonstrated using NADH peroxidase, an enzyme for which the active site cysteine sulfenic acid has been well characterized (*9, 10, 19, 20*). We therefore expect this reagent to be generally useful for the identification of cysteine sulfenic acid species present in a wide variety of other enzymatic and regulatory proteins.

MATERIALS AND METHODS

Materials. Mutant and wild-type AhpC proteins were isolated as previously described (*4, 7*). NBD-Cl and 5,5-dimethyl-1,3-cyclohexanedione (dimedone) were purchased from Aldrich. The standard buffer used in most experiments was 50 mM potassium phosphate with 1 mM EDTA at pH 7.0, unless noted otherwise.

Chemical Modification Procedures. Modifications of the sulfenic acid- and thiol-containing proteins were performed under anaerobic conditions similar to those described previously (Figure 1; *5*). Reduced or oxidized proteins were mixed with a small volume of NBD-Cl in dimethyl sulfoxide in the side arm of the anaerobic cuvette and incubated for 30–60 min at 25 °C in the dark (*15*). Modification of oxidized C165S AhpC with dimedone was performed under similar conditions except that a 100-fold excess of dimedone in 100% ethanol was used and the incubation was continued for 12 h at 25 °C (*12, 14*).

Recombinant NADH peroxidase, isolated in a partially-reduced state from *Escherichia coli* (*21*), was preincubated at room temperature with 1.5 equiv of H_2O_2 for 30 min, then diluted with buffer, and reconcentrated in a Centricon CM-30 to remove excess H_2O_2 prior to chemical modification.

[2] Cys-S-NBD denotes the product from the reaction of a cysteine thiol with NBD-Cl; Cys-S(O)-NBD denotes the product from the reaction of a cysteine sulfenic acid with NBD-Cl.

In other experiments, reduced protein was generated by the addition of 2 equiv of NADH and incubated until the absorbance decrease at 340 nm was no longer observed. For modification, 20 nmol of the anaerobic protein was mixed with guanidine HCl at pH 7.0 (2 M final concentration) quickly followed by incubation with NBD-Cl as described above. For all modified proteins, final spectra were taken after free NBD-Cl and/or FAD were removed by three cycles of concentration and redilution with standard buffer in a Centricon CM-30. An extinction coefficient of 13 000 M^{-1} cm^{-1} at 420 nm was used to quantitate the NBD-thiol product (*15*). Spectral scans were performed on Beckman DU7500 and Milton Roy Spectronic 3000 diode array spectrophotometers. Fluorescence spectra were recorded using an SLM Aminco Bowman Series 2 luminescence spectrophotometer.

Activity and Thiol Assays. All steady-state aerobic activity assays were performed in a stopped-flow spectrophotometer thermostated at 25 °C as previously described (*7*). AhpC wild-type and mutant proteins were in limiting concentrations (10 and 25 nM) while AhpF was in excess (1 μM). Assays were performed with the NBD-modified proteins before and after treatment with 2 equiv of DTT; proteins were isolated from free reagents using Sephadex G-50 chromatography and reconcentrated prior to assay. Thiol contents of AhpC proteins were measured spectroscopically as previously described (*5*).

Mass Spectrometry. ESIMS determinations were made on a VG Quattro II triple quadrupole mass spectrometer from Micromass in the GC-Mass Spectrometry Laboratory supported by NSF Grant 9414018 and Comprehensive Cancer Center Grant CA-12197 from the NIH. Excess reagents and buffer were removed and samples prepared as described earlier (*7*).

RESULTS

Spectral Characteristics of NBD-Cl-Treated AhpC Proteins. Each of the single (C46S, C165S) and double (C46,165S) mutants of AhpC were treated with NBD-Cl under anaerobic conditions with and without prior exposure to 1 equiv of H_2O_2 as was carried out previously for modifications with TNB (*7*). As shown in Figure 1, reduced C165S modified with NBD-Cl resulted in a new covalently-attached spectral species with an absorbance maximum at 420 nm consistent with previously-characterized thiol adducts with NBD-Cl (Cys-S-NBD; *15*); quantitation of this product indicated the presence of a single NBD-thiol adduct per polypeptide (0.94 ± 0.03; $N = 6$). Oxidized C165S generated by pretreatment with H_2O_2, on the other hand, gave an NBD-modified product (tentatively designated Cys-S(O)-NBD, see below) with an absorbance maximum at 347 nm (Figure 1), a spectral signature very similar to that of the free NBD-Cl ($\lambda_{max} = 343$ nm; *22*). Although we had no information about the extinction coefficient of this novel species, a comparison of the final spectra normalized to the same A_{280} suggested a very similar value for the two adducts ($\epsilon_{347} \sim 13\,400$ M^{-1} cm^{-1} for Cys-S(O)-NBD, assuming the same stoichiometry of incorporation). C46S was previously shown to be inactive in peroxidase assays and, similar to our previous finding, we detected only the formation of the thiol adduct with NBD (Cys-S-NBD) even following pretreatment of this mutant with a 10- or 100-fold excess of H_2O_2 (*7*), indicating that Cys165 remains in the thiol form under these conditions. As expected, the cysteine-deficient double mutant of AhpC, C46,165S, was unchanged on incubation with NBD-Cl either before or after exposure to H_2O_2. Wild-type AhpC was only reactive toward NBD-Cl if first reduced with DTT, and again only the NBD-thiol adduct was observed, although this time at a stoichiometry of two modifying groups per polypeptide.

Table 1: Mass Spectrometric Analyses of the Mutant (C165S and C46S) and Wild-Type AhpC Proteins[a]

protein	−NBD	+NBD[b]
wild-type		
reduced	20 615.8 ± 0.5	20 945.5 ± 0.9
oxidized	41 229.3 ± 1.9	ND[c]
C165S		
reduced	20 599.9 ± 0.6	20 765.2 ± 0.6
+1 equiv H_2O_2	20 649.1 ± 2.7	20 781.5 ± 3.1
+1 equiv CHP	20 648.1 ± 1.2	20 776.9 ± 2.4
C46S		
reduced	20 596.4 ± 0.7	20 762.5 ± 1.4
+1 equiv H_2O_2	20 598.2 ± 1.3	20 763.6 ± 2.5

[a] Mass spectrometry results are reported in atomic mass units (amu). [b] The expected added mass for NBD is 164 amu. [c] ND = not determined.

In other spectral studies with NBD-Cl, we confirmed that the C165S Cys-S-NBD conjugate was also fluorescent ($\lambda^{ex}{}_{max} = 422$ nm and $\lambda^{em}{}_{max} = 527$ nm) as has been well-established in other systems (*15*, *23*). In similar experiments with the C165S Cys-S(O)-NBD conjugate, no fluorescence of this product was observed. Attempts were also made to trap the sulfenic acid of the C165S mutant during turnover with the AhpF protein. Under these conditions, NBD-Cl reacted with reduced AhpF and produced a red product that absorbed around 560 nm, interfering with the formation and identification of the Cys-S(O)-NBD product; alternative products of NBD-Cl which possess unusual spectral features and form in the presence of excess reductants have previously been reported (*24*).

Given the possibility that the product of oxidation of the C165S mutant by an alkyl hydroperoxide would be a cysteine sulfenate ester (Cys-SOR) rather than a cysteine sulfenic acid (Cys-SOH) as expected from H_2O_2 oxidation, NBD-Cl modification of C165S was also carried out after 15 min pretreatment of the protein with 1 equiv of cumene hydroperoxide. Spectral characteristics of this NBD-modified product were identical with those of the product from the H_2O_2-treated protein, suggesting that Cys-SOH was generated in C165S upon oxidation of Cys46 by either peroxide substrate.

Mass Spectrometry of NBD-Modified AhpC Mutants. In order to aid in the identification of the NBD-modified products generated in the experiments described above, each of the samples before and after NBD-Cl treatment were analyzed by ESIMS. As shown in Table 1, NBD-modified C46S with or without pretreatment with H_2O_2 yielded products of nearly identical mass consistent with the expected value for a Cys-S-NBD conjugate of approximately 20 763 amu. The NBD-modified C165S mutant prior to peroxide treatment also showed a mass similar to that of C46S, while the NBD-modified product following H_2O_2 or cumene hydroperoxide treatment had a greater mass, consistent with the 16 amu difference expected if the oxygen of the sulfenic acid were retained in the final product (Figure 2). The product of oxidation of C165S by 1 equiv of H_2O_2 or cumene

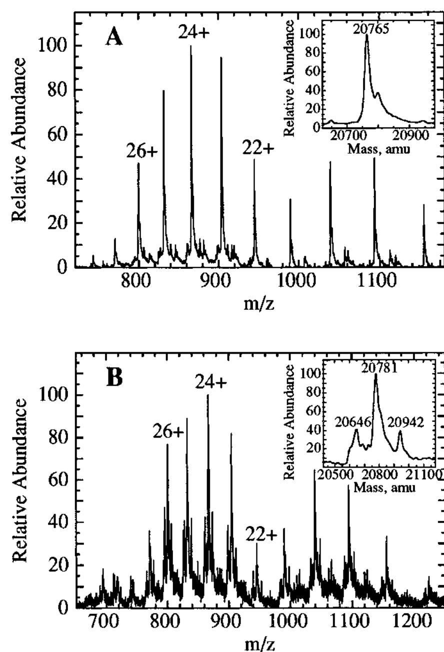


FIGURE 2: Electrospray mass spectra for NBD-modified products of reduced and oxidized C165S AhpC. NBD-modified C165S proteins (7−10 nmol) generated with or without pretreatment by 1 equiv of H_2O_2 were concentrated and rediluted five times with deionized water using Centricon CM-30 filtration units, generating final samples of about 400 μL containing 50% HPLC-grade acetonitrile and 1% formic acid. The capillary temperature was 80 °C, and the spray voltage was 3.5 kV. The mass spectrometer was calibrated in the high-MW range using myoglobin. Each panel shows the full ESIMS pattern of peaks with several of the peak charges labeled; the insets show a deconvolution of the series of peaks to give the final mass value(s). Panel A shows the mass spectrum obtained from a sample of the Cys-S-NBD conjugate of C165S; Panel B shows that for the Cys-S(O)-NBD conjugate. The two additional products detected in the Cys-S(O)-NBD sample correspond to over-oxidized protein which was not labeled by NBD (20 645.5 ± 3.3) and a small amount of Cys-S(O)-NBD product that was labeled with a second NBD (20 942.2 ± 1.8). Note that this latter product was present in amounts too small to be detected spectrally (Figure 1).

hydroperoxide in the absence of NBD-Cl treatment gave a mass indicating that the sulfenic acid had been oxidized to sulfonic acid once the protein was exposed to aerobic conditions (Table 1). The mass obtained for the NBD-Cl treated mutant of AhpC lacking both cysteine residues, C46,165S, was 20 583.1 ± 0.7 amu, indicating no reaction of this mutant with NBD-Cl as is consistent with the spectral data described above. Wild-type AhpC modified by NBD-Cl following pretreatment with DTT gave a mass consistent with the formation of two NBD-thiol adducts per polypeptide.

Activity and Thiol Assays with NBD-Modified Proteins. It has previously been shown that cysteine residues in AhpC are essential for the reduction of alkyl hydroperoxides during catalysis (*4, 7*). To determine the effect of NBD modification on active AhpC proteins and to assess the stability of the modified products, steady-state peroxidase assays were performed in the presence of excess AhpF. As shown in Table 2, the Cys-S(O)-NBD and Cys-S-NBD conjugates of C165S showed no apparent reaction in the peroxidase assays, while the untreated C165S had specific activities similar to those previously reported (*7*). Wild-type AhpC was also completely inactivated by NBD if reduced prior to treatment with the reagent. C46S showed no activity under any of the conditions used, although this result is relatively uninformative since this mutant was previously shown to be without peroxidatic activity under any conditions (*7*).

Table 2: Peroxidase Assays of Modified and Unmodified C165S, C46S, and Wild-Type AhpC Proteins before and after the Addition of DTT[a]

protein	−DTT	+DTT[b]
wild-type		
oxidized	325.9 ± 3.8	ND
+NBD-Cl	327.4 ± 10.7	ND
reduced	348.6 ± 11.4	ND
+NBD-Cl	<1%	343.2 ± 7.9
C165S	332.9 ± 4.7	ND[c]
+NBD-Cl	<1%	345.7 ± 11.4
+1 equiv of H_2O_2 + NBD-Cl	<1%	354.6 ± 12.3
+1 equiv of H_2O_2 + dimedone	<1%	<1%
C46S	<1%	ND
+NBD-Cl	<1%	<1%
+1 equiv of H_2O_2 + NBD-Cl	<1%	<1%

[a] Assays were performed as previously described (*7*); values are in nM of NADH oxidized s^{-1} nM^{-1} of protein and are reported as the mean ± standard error for at least three replicates. [b] Assays were performed after the addition of 5 mM DTT to remove NBD and isolation of the protein on a gel filtration column. [c] ND = not determined.

Reactivation of NBD-modified C165S and wild-type AhpC proteins by DTT treatment was also tested; previous results by others had suggested that Cys-S-NBD should be fully reactivated by this treatment (*23*), although we had no basis to predict the effect of this reductant on the sulfenic acid adduct (Cys-S(O)-NBD). On incubation of the NBD-modified C165S and wild-type proteins with a 5-fold excess of DTT and isolation of the proteins from the reagent by gel filtration chromatography, spectral scans showed the complete absence of the unique spectral properties attributed to the NBD adducts described above. This result held true even for the Cys-S(O)-NBD conjugate of C165S, suggesting that NBD is removed from the proteins whether or not the additional oxygen is present in the adduct. Both conjugates of C165S, Cys-S-NBD and Cys-S(O)-NBD, also regained full peroxidase activity on treatment with DTT and removal of the free reagents. NBD-modified wild-type AhpC was also restored to full activity by DTT in this manner. Thiol quantitation of NBD-modified proteins before and after DTT treatment also confirmed that DTT restored the thiol contents of the wild-type and C165S AhpC proteins to their values prior to NBD modification (approximately 2 and 1, respectively; *7*), clearly indicating that the target of NBD modification in each case was a cysteinyl residue (or cysteine sulfenic acid in the case of oxidized C165S).

Reactivity of the Stabilized Cys-SOH of NADH Peroxidase toward NBD-Cl. To explore the general utility of NBD-Cl as a trapping agent for cysteine sulfenic acids, an enzyme in which the Cys-SOH at the active site had been well-characterized was subjected to experiments similar to those described above. The major difference between techniques required to modify the Cys-SOH of the *E. faecalis* NADH peroxidase and those needed for modification of Cys-SOH in oxidized C165S of AhpC is the well-documented need for denaturation of the former protein to allow for modification of this active-site species (*19, 20*). As is consistent with our AhpC data, oxidized NADH peroxidase exposed to NBD-Cl under denaturing conditions gave an NBD-modified product with an absorbance maximum at 347 nm, while the reduced protein treated similarly exhibited an absorbance

maximum at 420 nm. Activity assays were clearly inappropriate due to the denaturing conditions required for modification of this protein.

Dimedone Modification of the Cys-SOH of C165S. An additional test for the generation of Cys-SOH on H_2O_2 or cumene hydroperoxide treatment of C165S was carried out using a well-characterized reagent, dimedone, as the trapping agent. This reagent reacts with sulfenic acids, but not with thiol derivatives, and does not exhibit any distinguishing visible absorbance properties on reaction with Cys-SOH. Measurement of catalytic activities showed that the reduced C165S and oxidized and reduced wild-type AhpC proteins incubated with dimedone still maintained full peroxidatic activity; these proteins were apparently not modified by dimedone. Oxidized C165S treated with dimedone, on the other hand, had no apparent activity, and the dimedone adduct was not removed by the addition of DTT as was expected from earlier work by Benitez and Allison (Table 2; *12*). The value obtained by mass spectrometric analysis for the oxidized C165S mutant modified with dimedone was 20 740.3 ± 1.4 amu, an exact match with the expected mass of 20 740 amu. Dimedone modification of oxidized C165S was also demonstrated to block further modification by NBD-Cl, an additional confirmation that Cys46-SOH is the target for modification by both of these reagents.

DISCUSSION

The major goals of this study were to definitively identify the putative cysteine sulfenic acid intermediate in the peroxidatic reaction mechanism of AhpC and to develop a spectral assay with the electrophilic reagent NBD-Cl that takes advantage of the nucleophilic character of the sulfenic acid. The AhpC mutant of choice for these studies was C165S, a single cysteine-containing protein which was previously shown to be fully active as a peroxidase and apparently able to stabilize the cysteine sulfenic acid (Cys46-SOH) generated on reaction with hydrogen peroxide (*7*). We have demonstrated herein that the NBD-modified product of oxidized C165S showed a complete loss of peroxidatic activity and exhibited an absorbance spectrum different from that of the reduced form of C165S modified with NBD. The different spectral characteristics of these NBD products correlated with the presence or absence of the sulfenic acid-derived oxygen as demonstrated by ESIMS; the sulfenic acid product, Cys-S(O)-NBD, exhibited an absorbance maximum at 347 nm while the thiol product, Cys-S-NBD, like others previously characterized (*15, 18, 23*), had a λ_{max} of 420 nm. The Cys46-SOH of C165S modified with dimedone, a commonly used sulfenic acid reagent, was no longer reactive toward NBD-Cl. The C46S mutant which was previously shown to be unreactive toward peroxides (*7*) yielded only the thiol conjugate on incubation with NBD-Cl before or after peroxide treatment. The double mutant lacking both cysteine residues (C46,165S) was completely unreactive toward NBD-Cl, and wild-type AhpC was only reactive toward NBD-Cl in its reduced form, further confirming that cysteinyl residues are the targets for NBD modification under these conditions.

Previous studies of proteins modified by NBD-Cl have taken advantage of the different absorbance and fluorescence properties of tyrosyl-, amino-, and thiol-NBD products (*15*−*18*). Our studies have now shown that the NBD-sulfenic acid conjugate has its own unique absorbance maximum, but no detectable fluorescence. The Cys-S(O)-NBD conjugate has proven to be relatively stable; the spectrum of the covalently-attached NBD remains unchanged for weeks on storage at −20 °C, although our mass spectrometric analyses were typically performed within 24 h of generating the conjugate. Efforts to isolate tryptic peptides from the modified protein were not successful, apparently due to instability of the adduct on incubation for several hours in the presence of trypsin and/or 2 M urea.

Our studies have clearly demonstrated the utility of NBD-Cl as both a thiol and sulfenic acid reagent capable of differentiating between the two oxidation states of sulfur through both spectral and mass spectrometric means. Studies with NADH peroxidase indicated the usefulness of NBD-Cl as a sulfenic acid reagent even in cases where the protein must first be denatured to promote modification. Spectral identification of the Cys-S(O)-NBD product was unambiguous in this case, although mass spectrometric analysis of this species was not attempted and could be complicated by the presence of the denaturant in these samples.

We have assumed that the stabilized product of the reaction of NBD-Cl with sulfenic acids is a sulfoxide derivative (Scheme 2), although we do not yet have empirical data to support such a suggestion. Modification of Cys-SOH with NBD-Cl takes advantage of the highly nucleophilic character of sulfenic acids; the sulfur atom is strongly nucleophilic due to the α-effect of the adjacent oxygen bearing two lone pairs of electrons, although the oxygen itself is also nucleophilic under the appropriate conditions (*11, 25*). Further efforts will be required to definitively identify which tautomer is stabilized following the NBD modification of Cys-SOH.

Our studies have confirmed and extended our previous characterization of the cysteinesulfenic acid generated on oxidation of the C165S mutant of AhpC by stoichiometric amounts of peroxide. It is now clear, for example, that exposure of the Cys-SOH to oxygen or excess peroxide leads to further oxidation of this residue to its sulfonic acid form (Cys-SO_3H), a species which is unreactive toward NBD-Cl (Table 1; Figure 2). Cumene hydroperoxide has also been shown to generate the same product as hydrogen peroxide on reaction with the active-site cysteine of C165S, a result which clearly identifies RO^- (presumably protonated by a proximal active site base) as the leaving group and rules out a formation of a Cys-SOR intermediate in this reaction. Taken together with our previous findings, these results strongly support a catalytic mechanism for the peroxidatic activity of AhpC which begins with attack of the nucleophilic thiolate of Cys46 on the −O−O− bond of the peroxide to displace the RO^- leaving group and form the enzyme-associated cysteine sulfenic acid (Cys46-SOH) followed in the wild-type enzyme by condensation of this intermediate with the proximal thiol(ate) of Cys165.

ACKNOWLEDGMENT

The authors thank Jeff Schmitt for suggesting NBD-Cl as a sulfenic acid modifying agent and E. J. Crane and Al Claiborne for their donation of *E. faecalis* NADH peroxidase for these studies.

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BI972191X