Identification and localization of a stable sulfenic acid in peroxide-treated tetrachlorohydroquinone dehalogenase using electrospray mass spectrometry

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Background: Tetrachlorohydroquinone dehalogenase catalyzes the reductive dehalogenation of tetrachlorohydroquinone to trichlorohydroquinone and then to 2,6-dichlorohydroquinone. This enzyme undergoes oxidative damage during purification which causes it to form aberrant products. The damage is reversible by treatment with dithiothreitol. Possible types of oxidative damage include an inappropriate disulfide bond, a cysteine sulfenic acid, or a methionine sulfoxide.

Results: Using electrospray liquid chromatography / mass spectrometry, we have demonstrated that oxidation of tetrachlorohydroquinone dehalogenase with H_2O_2 results in formation of a sulfenic acid at Cys13. Further oxidation to a sulfinic acid was also observed.

Conclusions: Oxidation of Cys13 to a sulfenic acid prevents the normal reductive dehalogenation reaction from being completed. This finding is consistent with previous work which suggested that Cys13 acts as a nucleophile during the conversion of tetrachlorohydroquinone to trichlorohydroquinone. The technique described for identification and localization of the cysteine sulfenic acid should be applicable to a wide variety of biological systems.

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Key words: glutathione S-transferase, sulfenic acid, tetrachlorohydroquinone dehalogenase

Received: **13 Aug 1996**
Revisions requested: **6 Sep 1996**
Revisions received: **2 Oct 1996**
Accepted: **2 Oct 1996**

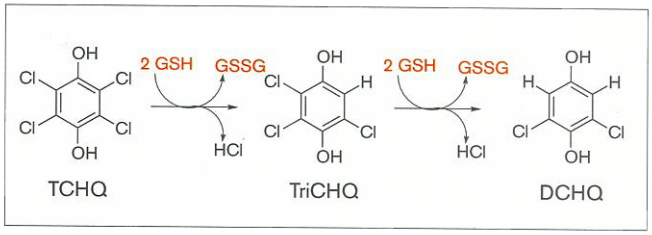
Chemistry & Biology October 1996, **3**:851–857

Introduction

Tetrachlorohydroquinone (TCHQ) dehalogenase is an enzyme from *Sphingomonas chlorophenolicus sp.* ATCC 39723 that is required for the degradation of pentachlorophenol, a widely used wood preservative [1]. This enzyme catalyzes the reductive dehalogenation of TCHQ to trichlorohydroquinone (TriCHQ) and then to 2,6-dichlorohydroquinone (DCHQ) (see Fig. 1). The reducing equivalents for each step are provided by two molecules of glutathione (GSH) [2]. In addition to these products, the purified enzyme forms substantial amounts of 2,3,5-trichloro-6-(*S*)-glutathionylhydroquinone (GS-TriCHQ) and an unidentified isomer of dichloro-(*S*)-glutathionylhydroquinone (GS-DCHQ). We have recently concluded that TCHQ dehalogenase undergoes oxidative damage during purification, resulting in a mixed population of damaged and undamaged enzymes [3]. Apparently the undamaged enzyme converts TCHQ to 2,6-dichlorohydroquinone (2,6-DCHQ) and the damaged enzyme converts TCHQ to GS-TriCHQ and GS-DCHQ (see Fig. 2).

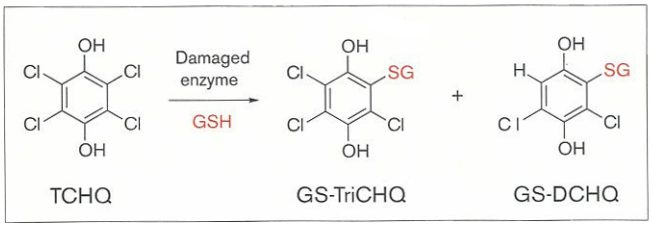
Since the exact location and nature of the oxidative damage is important to interpret the results of our mechanistic studies, we undertook to identify the oxidatively damaged residue in this enzyme. Our previous observations have shown that the oxidative damage can be reversed by dithiothreitol (DTT). Therefore, possible types of oxidative damage include an inappropriate disulfide bond, a cysteine sulfenic acid or a methionine sulfoxide. All of these are intriguing possibilities, since one of the two cysteines and at least two of the eight methionines in the enzyme are likely to be in or near the active site. We have recently found evidence that Cys13 acts as a nucleophile during the reductive dehalogenation reaction (see Fig. 3) [3], so Cys13 is a prime candidate for the oxidatively damaged residue. There is also a methionine nearby at position 15. Another methionine at position 10 is adjacent to the conserved serine residue (Ser11) that is believed to stabilize the thiolate of glutathione at the active site of enzymes in the theta class of the glutathione S-transferase superfamily [4]. (TCHQ

Figure 1



TCHQ dehalogenase catalyzes the reductive dehalogenation of TCHQ to TriCHQ and then to DCHQ.

Figure 2



Oxidatively damaged TCHQ dehalogenase converts TCHQ to GS-TriCHQ and GS-DCHQ.

dehalogenase has clear mechanistic similarities to, and sequence homology with, these theta-class enzymes [3].) Oxidation of any of these residues could perturb the structure and function of the active site. Here, we describe experiments that show that oxidative damage to TCHQ dehalogenase results in conversion of Cys13 in the active site of the enzyme to a sulfenic or sulfinic acid.

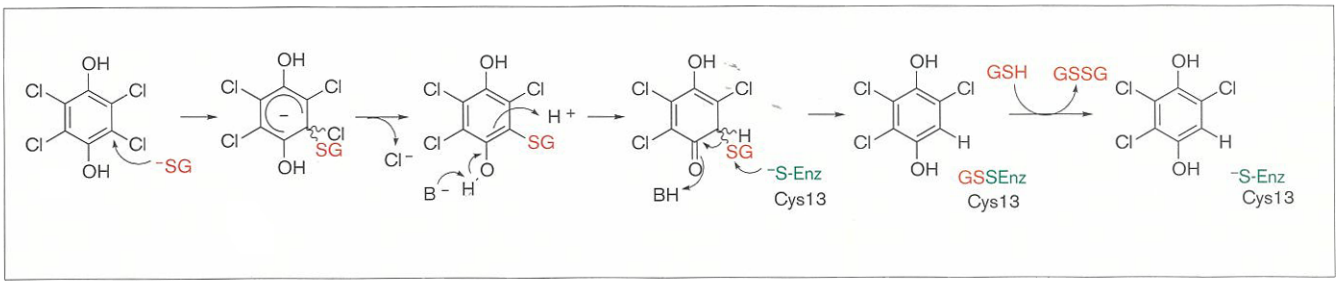
Sulfenic acids in biological systems

Sulfenic acids are important intermediates in many chemical reactions of thiols. They are the initial products formed during the oxidation of thiols to sulfonic acids and are also likely to be intermediates in the formation of disulfide bonds. Sulfenic acids are notoriously difficult to study because they are highly unstable. They readily condense to form thiosulfinates, which rapidly disproportionate to thiosulfonates and disulfides. Because of this high intrinsic reactivity, only a few stable sulfenic acids have been synthesized. In these compounds, reaction of the labile sulfenic acid moiety is prevented either by steric constraints or by intramolecular hydrogen bonding [5].

Because of the ubiquitous occurrence of cysteines in proteins, sulfenic acids are also of great interest in biological systems. The existence of stable sulfenic acids in proteins has been considered for over forty years [6]. At least two enzymes, Streptococcal NADH peroxidase [7] and Streptococcal NADH oxidase [8], seem to use cysteine sulfenic acids as an integral part of the catalytic cycle. It has also been proposed that cysteine sulfenic acids are involved in the redox regulation of certain transcription factors [9]. On the other hand, formation of cysteine sulfenic acids is often a consequence of oxidative damage. Exposure of proteins to oxidizing agents such as peroxide [10] and nitric oxide (NO•) [11] results in the formation of sulfenic acids, and adventitious oxidation of cysteine residues to cysteine sulfenic acids occurs during purification of numerous proteins. The consequences of this oxidative damage depend upon the location of the cysteine residue and its role in maintaining protein structure and activity. Formation of cysteine sulfenic acids in the active site can alter or abolish enzymic activity. Papain, a cysteine protease, is inactivated by conversion of the active-site cysteine to a sulfenic acid [12]. Glyceraldehyde 3-phosphate dehydrogenase is converted into an acyl phosphatase by oxidation of the active site cysteine residue [13]. Less dramatic effects resulting from oxidation of cysteine residues in remote sites have been found in several purified proteins, including transcortin [14], bovine seminal ribonuclease [15], and *p*-hydroxybenzoate hydroxylase [16]. In bovine seminal ribonuclease, the proposed cysteine sulfenic acid causes misfolding of the enzyme, but activity is retained.

Unfortunately, evidence for the existence of stable sulfenic acids in proteins has largely been indirect, relying on differences in the number of titratable thiol residues in a protein before and after treatment with reagents such as DTT or arsenite, which reduce sulfenic acids to thiols [12]. Until recently, the most direct analysis of a sulfenic acid in a protein was provided by Benitez and Allison [17], who derivatized the active-site sulfenic acid of oxidized glyceraldehyde 3-phosphate with ^{14}C-dimedone, isolated the radiolabeled peptide, and determined its sequence. However, because ^{14}C-dimedone is not commercially available, this technique has not been used by other workers. Recently, a crystal structure of Enterococcal NADH peroxidase

Figure 3



Postulated mechanism of TCHQ dehalogenase. Cys13 attacks the sulfur atom of the tautomer of GS-TriCHQ at the active site.

containing a cysteine sulfenic acid was reported [18]. Although X-ray crystallography provides the best information about the structure of the cysteine sulfenic acid and the environment that stabilizes it, the large amounts of time and material required for this technique limit the application of this method. Here, we report a method using electrospray liquid chromatography / mass spectrometry (LC/MS) that will allow identification and localization of cysteine sulfenic acids in most proteins using very small amounts of material.

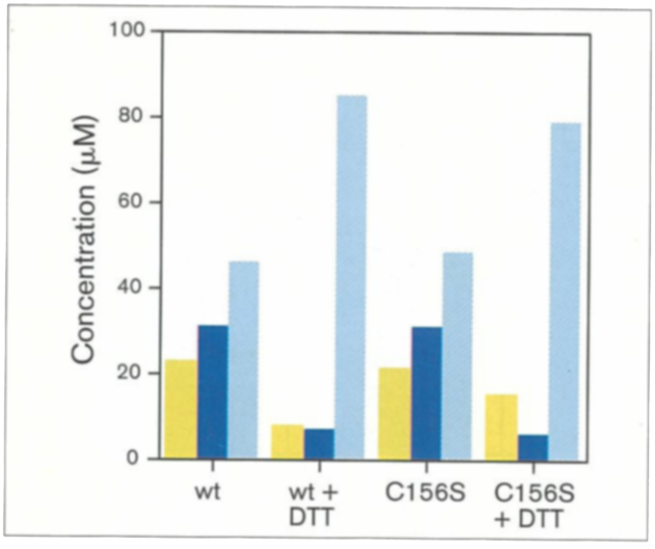
Results and discussion

To enhance our ability to detect the oxidative damage to TCHQ dehalogenase, we treated the enzyme with an equimolar amount of H_2O_2 to increase the fraction of the enzyme that was oxidized. (Higher concentrations of H_2O_2 led to extensive damage to the protein, as judged by the appearance of multiple peaks in the electrospray LC/MS data, all of which had higher molecular weights than the native protein and had apparently incorporated numerous oxygen atoms at various sites.)

The oxidative damage is not an intramolecular disulfide bond

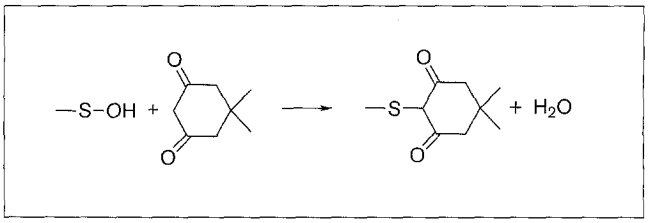
To explore the possibility that oxidative damage to TCHQ dehalogenase involves formation of an intramolecular disulfide bond between the two cysteine residues (Cys13 and Cys156), we used electrospray LC/MS to analyze a tryptic digest of H_2O_2-treated TCHQ dehalogenase. The protein was treated with 4-vinylpyridine to trap any free sulfhydryl groups prior to digestion with trypsin. We found no evidence for a dipeptide linked by a disulfide bond between Cys13 and Cys156 (data not shown). In addition, we analyzed the products formed by oxidation of the Cys156→Ser (C156S) mutant TCHQ enzyme. As shown in Figure 4, the susceptibility of the C156S mutant to oxidative damage is identical to that of the wild-type enzyme, as judged by the formation of glutathione conjugates from TCHQ and the ability of DTT to reverse the oxidative damage. Thus, Cys156 cannot be involved in the oxidative damage that alters the reactivity of the enzyme.

Figure 4



The susceptibility of the C156S mutant to oxidative damage is identical to that of the wild-type enzyme. Distribution of products at the completion of a reaction containing 100 μM TCHQ, 0.02 % ascorbate, 1 mM GSH, either wild-type or C156S TCHQ dehalogenase, and, where indicated, 25 mM DTT. Yellow, GS-DCHQ; dark blue, GS-TriCHQ; cyan, DCHQ.

Figure 5



Treatment of a sulfenic acid with dimedone forms a stable thioether adduct.

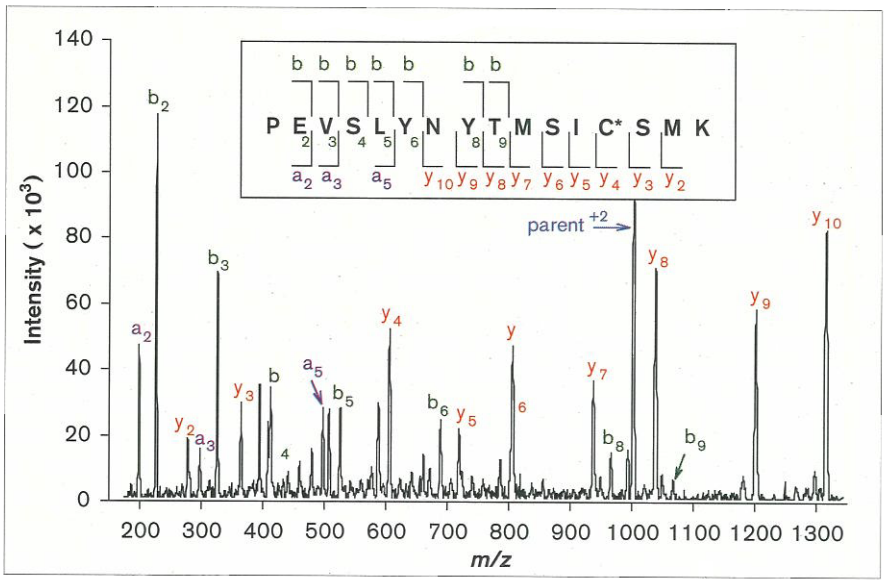
During the course of this work, we observed by both LC/MS and non-reducing SDS polyacrylamide gel electrophoresis (SDS-PAGE) that the wild-type enzyme forms small amounts of dimer, while the C156S mutant does not (data not shown), suggesting that Cys156 is on the surface of the enzyme and can form intermolecular disulfide bonds. To eliminate this complicating factor, all further experiments were carried out with the C156S mutant.

Detection of a cysteine sulfenic acid by electrospray LC/MS

Peroxide-treated TCHQ dehalogenase was treated with dimedone to trap as a stable thioether adduct any sulfenic acid that might be present (see Fig. 5). The enzyme was then digested with trypsin and subjected to electrospray LC/MS analysis. The results of the LC/MS analysis show clear evidence for a dimedone adduct of Cys13. The expected mass for the amino-terminal peptide is 1866 amu. In a sample of enzyme treated with H_2O_2 and dimedone, a peptide with a mass of 2004 amu was detected. The increase in mass is exactly that expected for a dimedone adduct with a cysteine. To confirm this finding, we subjected this peptide to sequencing by LC/MS/MS. The pattern of daughter ions generated by fragmentation of the peptide from either end confirms the identity of the dimedone adduct of the amino-terminal peptide (see Fig. 6). In particular, the difference in mass between the y_3 and y_4 fragments (241 amu), shows that the cysteine residue has been replaced with the expected dimedone adduct.

The possibility of oxidation products involving the methionine residues in the amino-terminal peptide (at

Figure 6



LC/MS/MS confirms the presence of an adduct between dimedone and Cys13. "C*" indicates the modified cysteine residue. Nomenclature for identification of fragments is as follows. Cleavage of the amide bond (the most common cleavage site) results in amino-terminal fragments, which are designated with a 'b', and carboxy-terminal fragments, which are designated with a 'y'. The subscript indicates the number of amino-acid residues in the fragment. Cleavage of the bond between the alpha carbon and the carbonyl carbon results in amino-terminal fragments, which are designated with an 'a', and carboxy-terminal fragments, which are designated with an 'x'. (No 'x' fragments are labeled in the figure.)

positions 10 and 15) was also addressed by electrospray LC/MS, but no evidence for such products was found.

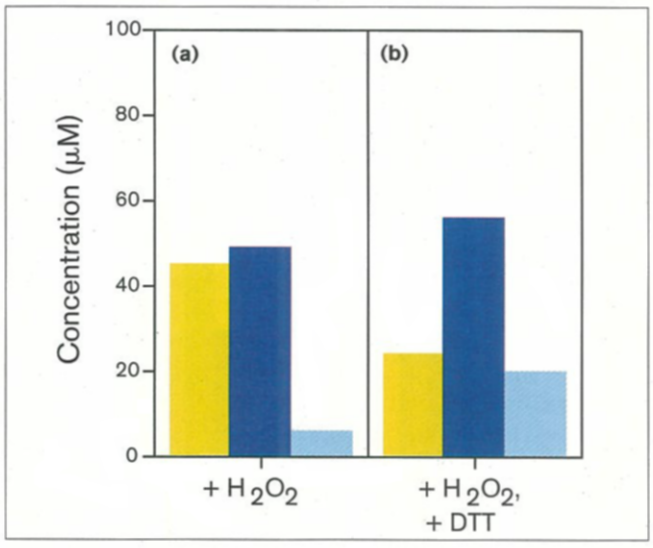
Repair of the peroxide-induced damage by DTT is only partially successful

We have previously observed that the unintentional oxidative damage to TCHQ dehalogenase that occurs during purification can be almost completely reversed by treatment with 25 mM DTT (see Fig. 4). In contrast, treatment of the H_2O_2-oxidized enzyme with 25 mM DTT resulted in only partial repair of the oxidative damage, as judged by the distribution of products (see Fig. 7). The fact that glutathione conjugates are still made in the presence of DTT suggests that some of the enzyme has undergone additional oxidative damage that is not reversible by DTT. Indeed, LC/MS analysis of the sample of oxidized enzyme showed that, in addition to enzyme molecules bearing a dimedone moiety, some enzyme molecules containing two additional oxygen atoms were present (data not shown). Digestion of this enzyme with trypsin gave a peptide with a mass of 1898 amu (corresponding to the mass of the native amino-terminal peptide plus 32 amu) which was sequenced by LC/MS/MS (see Fig. 8). The difference in mass between the y_3 and y_5 fragments (248 amu) shows that the cysteine residue has been converted to a sulfinic acid. (The peak due to the y_4 fragment is obscured beneath another prominent peak in the spectrum.) Oxidation of Cys13 to a sulfinic acid would be expected to cause the same functional defect as oxidation to a sulfenic acid, but sulfinic acids are not reduced by DTT [19]. Thus, treatment of the enzyme with H_2O_2 under these conditions results in formation of both the cysteine sulfenic acid and the cysteine sulfinic acid; the fact that the oxidative damage is only partially repaired by DTT can be attributed to the presence of some enzyme molecules containing cysteine sulfinic acid at position 13.

Relevance to the mechanism of TCHQ dehalogenase

The catalytic mechanism that we have recently proposed for TCHQ dehalogenase [3] is shown in Figure 3. Cys13 is

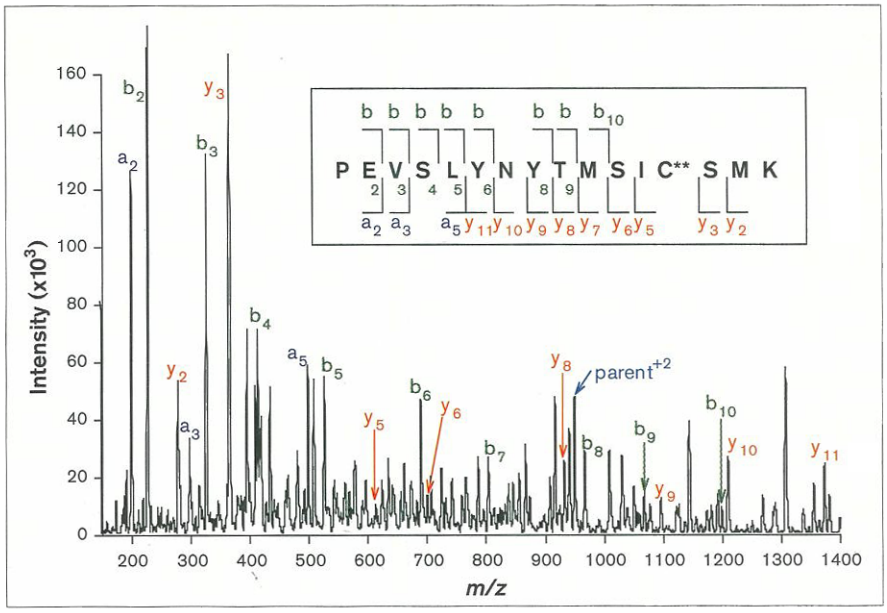
Figure 7



Oxidative damage to H_2O_2-treated C156S TCHQ dehalogenase is only partially reversed by treatment with DTT. Distribution of products at the completion of a reaction containing **(a)** 100 μM TCHQ, 0.02 % ascorbate, H_2O_2-treated C156S TCHQ dehalogenase and **(b)** the same reagents plus 25 mM DTT. Yellow, GS-DCHQ; dark blue, GS-TriCHQ; cyan, DCHQ.

Figure 8

LC/MS/MS of the amino-terminal peptide of oxidized TCHQ dehalogenase confirms the presence of a cysteine sulfinic acid at position 13. C** indicates the cysteine sulfinic acid. Nomenclature for identification of fragments is given in the legend to Figure 7.



believed to attack the sulfur atom of the tautomer of GS-TriCHQ at the active site. Conversion of Cys13 to a sulfenic acid apparently abolishes the ability of the enzyme to catalyze this step, as the products formed by the oxidized enzyme can be rationalized based upon the expected decomposition of the unstable tautomer of GS-TriCHQ, which is the last intermediate that can form at an active site that lacks a functional nucleophile at position 13. (The expected pathways for decomposition of this intermediate are described in detail in [3].) This conclusion is supported by the fact that the Cys13→Ser mutant enzyme [3] forms identical products. The inability of the oxidized enzyme to complete the reaction could be due to either or both of the following two factors: a decrease in the nucleophilicity of the sulfenic acid relative to a thiol, and misplacement of the nucleophile in the active site caused by the increased bulk of the sulfenic acid.

It has previously been postulated that sulfenic acids in proteins will only be stable if they are isolated from other sulfenic acids and reactive nucleophiles such as thiols [9,12]. This explanation is probably valid in this case. A crystal structure of TCHQ dehalogenase has not been solved, but the crystal structure of the blowfly glutathione S-transferase (to which TCHQ dehalogenase has about 20 % sequence identity (P.C. Babbitt, unpublished data)) has been reported [20]. Although the sequence identity between TCHQ dehalogenase and the glutathione S-transferases is rather low, the clear mechanistic similarities between the enzymes suggest that there may also be global structural similarities. The blowfly glutathione S-transferase uses a serine at position 9 to stabilize the thiolate of glutathione at the active site [21]. Ser9 is found in a deep cleft in this enzyme. The analogous serine residue in TCHQ dehalogenase is Ser11. Cys13 is only two residues away from Ser11, so it is likely to be also located in the bottom of the active-site cleft. Experimental support for this hypothesis is provided by the observation that the oxidative damage to TCHQ dehalogenase that occurs during purification can be repaired by small thiol reagents such as DTT and β-mercaptoethanol, but not by GSH (D. McCarthy and S.D.C., unpublished data). The thiol moiety of GSH apparently cannot reach the cysteine sulfenic acid, either because GSH is relatively large or because its position is fixed by binding interactions in the active site.

Significance

To our knowledge, this is the first use of electrospray LC/MS to identify and localize a sulfenic acid in a protein. Previous techniques have had severe limitations. Thiol titrations can be used to detect a change in the number of thiols when the enzyme is treated with reagents that reduce sulfenic acids, but they generally do not allow localization of the sulfenic acid. Localization techniques that involve further oxidation to the sulfonic acid result in a loss of definitive information about the oxidation state of the putative sulfenic acid. The most informative technique, X-ray crystallography, is limited by the requirement for large amounts of protein in the form of diffraction-quality crystals. The technique described here, which should be generally applicable to many biological systems, allows identification and localization of cysteine sulfenic acids using only microgram amounts of protein.

Materials and methods

Materials

TCHQ was purchased from Kodak (Rochester, NY). Dimedone, GSH and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) were purchased from Sigma (St. Louis, MO). Dithiothreitol, formic acid (88 %) and hydrogen peroxide (30 %) were obtained from Fisher Scientific (Pittsburgh, PA). Ascorbic acid was obtained from Aldrich (Milwaukee, WI).

Protein expression and purification

Escherichia coli strain JM109 was made competent by treatment with $CaCl_2$ and transformed with the plasmid pJD01 [22], containing the TCHQ dehalogenase gene under control of the *tac* promoter. Transformants were selected on LB-ampicillin plates. Cultures were started from individual colonies and grown 6–8 h at 37 °C in 2 ml of 2YT media (10 g l^{-1} tryptone, 16 g l^{-1} yeast extract, 5 g l^{-1} NaCl) in the presence of 100 μg ml^{-1} ampicillin. Four-liter flasks containing one liter of 2YT media were inoculated with 1 ml of starter culture and grown overnight at 37 °C with shaking but without induction by IPTG, as induction was found to be unnecessary. Cells were harvested by centrifugation for 10 min at 8000 x g at 4 °C. The cell pellet was washed with 0.9 % NaCl and then resuspended in 30 ml of lysis buffer (20 mM Tris-HCl, pH 8.0 containing 5 mM EDTA, 5 mM DTT and 0.1 mM AEBSF). Lysozyme (15 mg) and DNase (2 mg) were added and the cell suspension was incubated with shaking at room temperature for 30 min. The cells were broken by passage three times through a French press at >12 000 psi. Inclusion bodies containing TCHQ dehalogenase were isolated from the cell lysate by centrifugation at 12 000 x g at 4 °C for 30 min. The supernatant was discarded and the pellet was washed with lysis buffer. The inclusion bodies were solubilized in 30 ml of 20 mM Tris-HCl, pH 8.0, containing 8 M urea, 10 mM DTT and 1 mM EDTA. Insoluble material was removed by centrifugation at 12 000 x g for 30 min at 4 °C. The solubilized inclusion bodies were typically >80 % TCHQ dehalogenase as judged by SDS-PAGE. The solubilized inclusion bodies were dialyzed against 4 liters of 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 1 mM DTT, overnight at 4 °C with two changes of dialysis buffer. The dialyzed material was then loaded onto a Pharmacia Mono Q column (1 cm x 10 cm) equilibrated with the dialysis buffer. After washing the column with 3 column volumes of equilibration buffer, the protein was eluted with a gradient of 0–0.5 M NaCl in equilibration buffer. Fractions containing ≥ 95 % TCHQ dehalogenase, as determined by SDS-PAGE, were pooled and concentrated to ≤ 2ml in a Centricon 10 (Amicon). This concentrate was loaded onto a Superdex 200 gel filtration column (2 cm x 60 cm) equilibrated with 10 mM Tris-HCl, pH 8.0 containing 1 mM DTT and 1 mM EDTA. Fractions containing pure enzyme were pooled, concentrated with a Centricon 10, and stored at 4 °C. A typical final yield is 5–10 mg l^{-1} of culture at ≥ 99 % purity.

Activity assays

TCHQ dehalogenase was assayed as described previously [3].

Hydrogen peroxide and dimedone treatment

TCHQ dehalogenase (100 μM) was treated with 100 μM hydrogen peroxide in a total volume of 20 μl of 20 mM Tris-HCl, pH 7.0, containing 1 mM EDTA, for 10–30 min at 25 °C. Excess H_2O_2 was removed by adding 2 units of catalase (1 μl of 2000 units ml^{-1}) and incubating for 10 min at 25 °C.

Derivatization with dimedone and 4-vinylpyridine

For derivatization of sulfenic acids with dimedone, protein samples were treated with a 100-fold molar excess of dimedone and incubated for 1–2 h at 25 °C. For derivatization of free thiols with 4-vinyl pyridine, protein samples were purged and evacuated on an N_2/vacuum line four times, and 4-vinyl pyridine was added to a final concentration of 50 mM. The samples were incubated under N_2 in the dark at room temperature for 1h. The protein was precipitated by addition of nine volumes of cold acetone. The pellets were resuspended in 20 μl of 8 M urea containing 0.2 M Tris, pH 8.0 and then diluted to a final volume of 100 μl with 100 mM Tris, pH 8.0, prior to protease digestion.

Electrospray LC/MS and LC/MS/MS

TCHQ dehalogenase (treated with H_2O_2 and dimedone or 4-vinyl pyridine as described above) was digested with either TPCK-treated trypsin or endoprotease Lys C (Sigma) in 20 mM Tris-HCl, pH 7.0, containing 1 mM EDTA, for 1–2 h at 25 °C. The protease concentration was 100 nM. Reactions were quenched by addition of 1/10th volume of 10 % formic acid. LC/MS analysis of the resulting peptides was carried out by injecting 100–200 pmoles of digested enzyme onto a 500 μm capillary column packed with Vydac C18 resin and equilibrated with 0.1 % formic acid. After washing with 200 μl of 0.1 % formic acid, the column was connected directly to a triple quadrupole mass spectrometer (API-III, Sciex, Thornhill, ON, Canada) equipped with a nebulization-assisted electrospray ion source and a high pressure collision cell. The ion spray needle was held at a potential of 4.6 kV with an orifice voltage of 75 V. Peptides were eluted from the column into the mass spectrometer with a 1 % min^{-1} acetonitrile gradient. MS/MS experiments were performed on-line by selecting the desired ion prior to collision-induced fragmentation in the collision cell. Argon at a density of (3.0–3.5) x 10^{14} atoms cm^3 was used as the collision gas. Data were interpreted with programs supplied with the Sciex instrument (MacSpec and MacBioSpec).

Acknowledgements

We thank Professor Katheryn Resing and Dr Donna Louie for providing training on the LC/MS instrument. This research was supported by the National Science Foundation (Grant # MCB-9406909) and the Cooperative Institute for Research in Environmental Sciences at the University of Colorado.

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