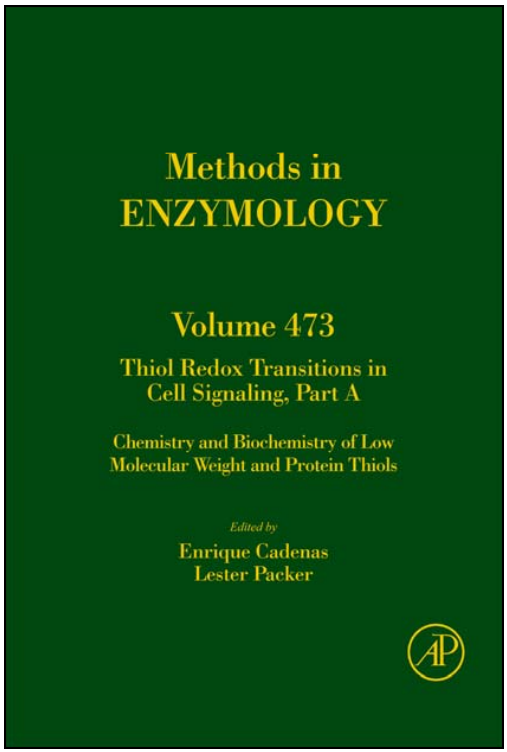

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CHAPTER THREE

Use of Dimedone-Based Chemical Probes for Sulfenic Acid Detection: Evaluation of Conditions Affecting Probe Incorporation into Redox-Sensitive Proteins

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Abstract

Sulfenic acids, formed as transient intermediates during the reaction of cysteine residues with peroxides, play significant roles in enzyme catalysis and regulation, and are also involved in the redox regulation of transcription factors and other signaling proteins. Therefore, interest in the identification of protein sulfenic acids has grown substantially in the past few years. Dimedone, which specifically traps sulfenic acids, has provided the basis for the synthesis of a novel group of compounds that derivatize 1,3-cyclohexadione, a dimedone analogue, with reporter tags such as biotin for affinity capture and fluorescent labels for visual detection. These reagents allow identification of the cysteine sites and proteins that are sensitive to oxidation and permit identification of the cellular conditions under which such oxidations occur. We have shown that these compounds are reactive and specific toward sulfenic acids and that the labeled proteins can be detected at high sensitivity using gel analysis or mass spectrometry. Here, we further characterize these reagents, showing that the DCP-Bio1 incorporation rates into three sulfenic acid containing proteins, papaya papain, *Escherichia coli* fRMsr, and the *Salmonella typhimurium* peroxiredoxin AhpC, are significantly different and, in the case of fRMsr, are unaffected by changes in buffer pH from 5.5 and 8.0. We also provide protocols to label protein sulfenic acids in cellular proteins, either by *in situ* labeling of intact cells or by labeling at the time of lysis. We show that the addition of alkylating reagents and catalase to the lysis buffer is critical in preventing the formation of sulfenic acid subsequent to cell lysis. Data presented herein also indicate that the need to standardize, as much as possible, the protein and reagent concentrations during labeling. Finally, we introduce several new test or control proteins that can be used to evaluate labeling procedures and efficiencies.

1. Introduction

Cysteine sulfenic acids in proteins are formed upon reaction of an activated cysteinyl residue with oxidants such as hydrogen peroxide, hydroperoxides, hypochlorous acids, or peroxynitrite (Poole *et al.*, 2004; Reddie and Carroll, 2008). This chemistry occurs, and can be important for modulating biological outcomes (Michalek *et al.*, 2007; Oshikawa *et al.*, 2010), during many receptor-mediated cell signaling processes and as a consequence of oxidative injury occurring due to environmental insults or pathogenic processes (Poole *et al.*, 2004). Thus, development of comprehensive (or even partial) lists of *bona fide* oxidation-sensitive sites in proteins, as well as cellular conditions under which such oxidation sites are engaged, will be critical to better inform biochemical and cellular studies on the consequences of oxidation at specific sites in target proteins and to enhance our understanding of the features characteristic of oxidation-sensitive cysteine sites (Salsbury *et al.*,

2008). At the protein level, the sulfenic acid moiety may be generated as a catalytic or regulatory species or may be the result of an adventitious oxidation with or without structural and/or functional consequences. The development of chemical tools to identify oxidation sites is an important first step toward determining the role that these oxidation events play in modulating protein activity, and ultimately, cellular processes.

Several chemical approaches have been used to evaluate sulfenic acid formation in pure proteins (Allison, 1976; Poole and Ellis, 2002; Turell *et al.*, 2008); the most promising approach for directly and irreversibly modifying sulfenic acids within proteins for proteomics-level analyses has been through use of 5,5-dimethyl-1,3-cyclohexanedione (dimedone), an alkylating agent specific for cysteine sulfenic acid (Allison, 1976; Poole *et al.*, 2005, 2007) or analogues thereof to chemically trap such species (Fig. 3.1) (Poole and Nelson, 2008). This strategy provides new, powerful tools to investigate sulfenic acid formation in proteins. A series of reporter-linked or -linkable, sulfenic acid-directed labeling reagents have been generated by our group and others based upon dimedone or 1,3-cyclohexadione (Fig. 3.1), the latter of which lacks the two methyl groups attached to the ring of dimedone (Poole *et al.*, 2005, 2007; Charles *et al.*, 2007; Leonard *et al.*, 2009; Reddie *et al.*, 2008). Reagents that incorporate a biotin affinity tag or fluorescent groups into a 1,3-cyclohexadione moiety via a linker (Poole *et al.*, 2005, 2007) were used in this work and are shown in Fig. 3.2.

Reactivity of these reagents with protein sulfenic acids is determined in part by the accessibility and stability of the sulfenic acid species at each site. Moreover, within cells, "stability" of the sulfenic acid modification is significantly influenced by the local environment of the oxidized cysteine,

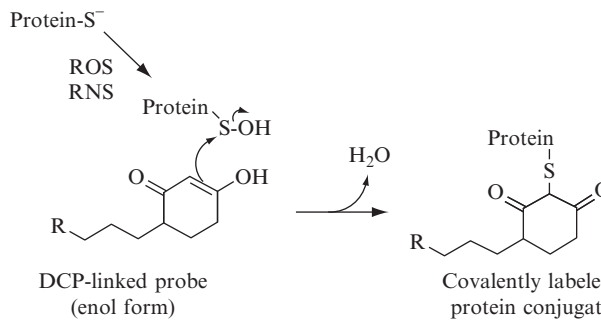

Figure 3.1 Reaction scheme for labeling protein sulfenic acids with DCP-linked probes. Protein thiolates (R–S–), which are susceptible to oxidation by reactive oxygen and nitrogen species (ROS and RNS, respectively) generate sulfenic acids (R–SOH), which can then be labeled by the probes that are synthesized using the reactive 1,3-cyclohexadione core of dimedone.

its tendency to react with other oxidants to form further oxidized cysteinyl moieties (i.e., sulfinic or sulfonic acids), and its accessibility to other thiol groups (i.e., cysteine or glutathione) that can react to form a disulfide bond. Thus, rapid "trapping" of sulfenic acids in proteins with alkylating chemical probes is of great advantage for detecting and identifying these species, even though only substoichiometric amounts of label would ever likely be incorporated into given proteins due to the generally transient nature of the modification. Reliable quantitative measurements based on the extent of probe incorporated are likely to be difficult to achieve, though large variations in oxidation for individual cellular proteins may be observable across samples within the same experimental set.

Evaluation of the reactivity of one of the most useful sulfenic acid probes, DCP-Bio1, toward pure proteins is the subject of the first part of this chapter. The second part provides protocols for labeling oxidized proteins within the cell and introduces several new tests or control proteins for evaluating labeling procedures and efficiencies. An accompanying chapter (Nelson *et al.*, 2010) addresses the use of various approaches for detecting and identifying oxidized proteins and specific sites of oxidation once probes have been incorporated.

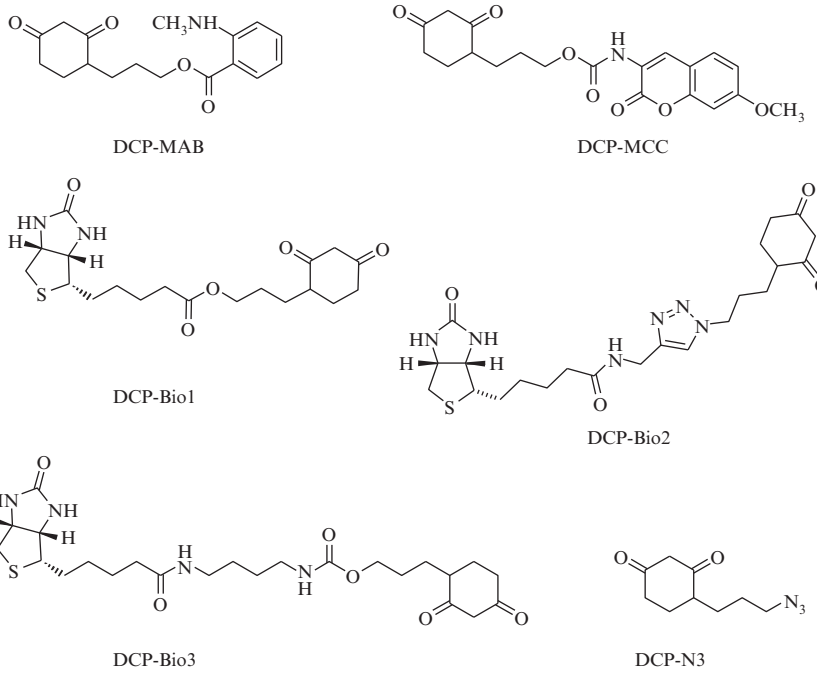

Figure 3.2 (*Continued*)

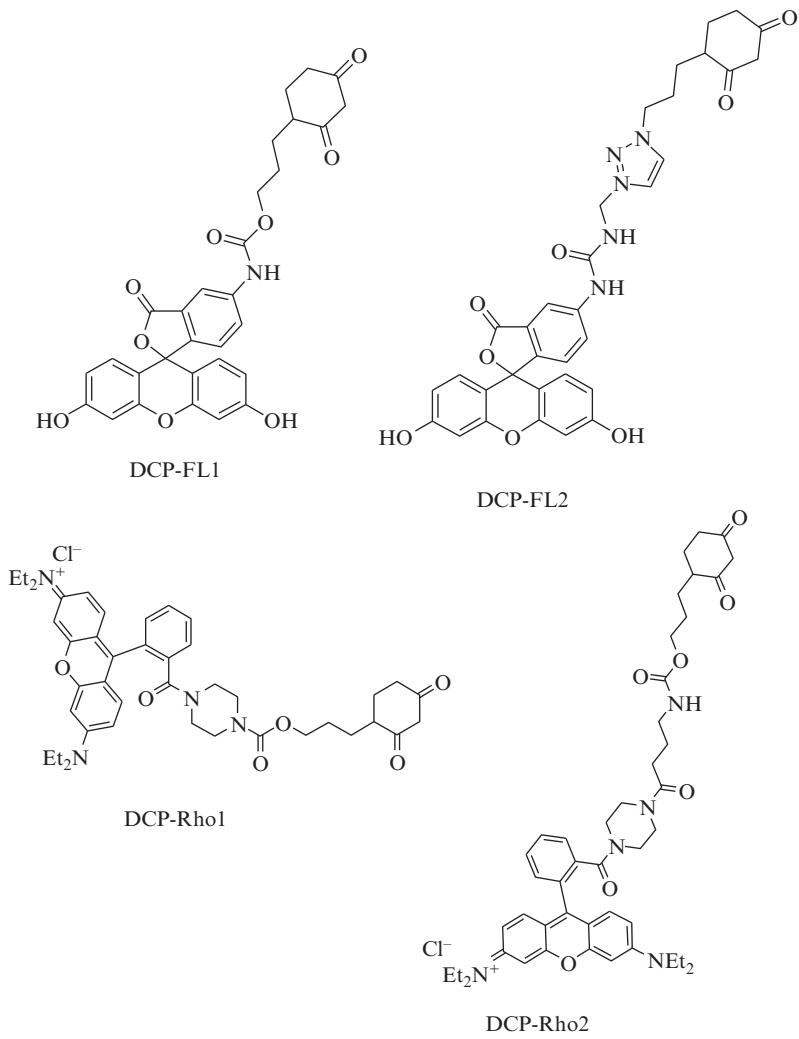

Figure 3.2 **Structures and shortened names for the DCP-linked, sulfenic acid-reactive probes used in the present work.**

2. Materials

2.1. Solutions

1. 100 m*M* Diethylene triamine pentaacetic acid (DTPA) in 1 *M* sodium hydroxide

2. Potassium phosphate buffers (5, 25, and 50 m*M*), pH 7.0, 100 μM DTPA
3. 50 m*M* Tris–HCl, pH 8.0, 100 μM DTPA
4. 50 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.3, 100 μM DTPA
5. 100 m*M* Methionine sulfoxide (racemic mixture) in 5 m*M* potassium phosphate, pH 7.0, 100 μM DTPA
6. 30% (~10 *M*) Hydrogen peroxide (H_2O_2)
7. 100 m*M* 1,4-Dithio-DL-threitol (DTT), 154.2 g/mol
8. Cell lysis buffer: 50 m*M* Tris base, pH 7.5 containing 100 m*M* sodium chloride, 100 μM DTPA, 20 m*M* β-glycerophosphate, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.5% NP-40, and 0.5% Triton-X-100
9. Phosphate-buffered saline (PBS); 100 m*M* sodium phosphate, 150 m*M* sodium chloride, pH 7.2
10. Sinapinic acid (20 mg) in 0.3% trifluoroacetic acid, 50% acetonitrile. Since this is a saturated solution, centrifuge prior to using.

2.2. Chemical modification agents

1. *N*-Ethylmaleimide (NEM), 125.13 g/mol
2. Iodoacetamide (IAAm), 184.96 g/mol
3. 3-(2,4-Dioxocyclohexyl)propyl 2-(methylamino)benzoate (DCP-MAB), 303.35 g/mol (Poole *et al.*, 2005)
4. 3-(2,4-Dioxocyclohexyl)propyl 7-methoxy-2-oxo-2H-chromen-3-ylcarbamate (DCP-MCC), 387.38 g/mol (Poole *et al.*, 2005)
5. Fluoresceinamine-5′-*N*-[3-(2,4-dioxocyclohexyl)propyl)]carbamate (DCP-FL1), 543.5 g/mol (Poole *et al.*, 2007)
6. Fluoresceinamine-5′-*N*-[3-((1-(3-(2,4-dioxocyclohexyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl]-urea (DCP-FL2), 623.6 g/mol (Poole *et al.*, 2007)
7. (DCP-Bio1), 396.5 g/mol (Poole *et al.*, 2007)
8. 5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)-*N*-((1-(3-(2,4-dioxocyclohexyl)propyl)-1*H*-1,2,3-triazol-4-yl) methyl)pentanamide (DCP-Bio2), 476.6 g/mol (Poole *et al.*, 2007)
9. 3-(2,4-Dioxocyclohexyl)propyl 4-(5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamido)butylcarbamate (DCP-Bio3), 510.7 g/mol (Poole *et al.*, 2007)
10. Rhodamine B [4-[3-(2,4-dioxocyclohexyl)propyl]carbamate]piperazine amide (DCP-Rho1), 707.9 g/mol (Poole *et al.*, 2007)
11. Rhodamine B 3-(2,4-dioxocyclohexyl)propyl 4-oxo-4-(piperazin-1-yl)butylcarbamate (DCP-Rho2), 793.0 g/mol (Poole *et al.*, 2007)

12. 4-(3-Azidopropyl)cyclohexane-1,3-dione, (DCP-N3), 195.1 g/mol, generated by deprotection of 3-ethoxy-6-(3-azidopropyl)-cyclohex-2-enone (Poole *et al.*, 2007) by treatment with 3 *M* HCl

2.3. Proteins

1. Catalase (2000 units/ml, Sigma) in 50 m*M* Tris–HCl, pH 7.5, 100 μM DTPA.
2. *Salmonella typhimurium* of AhpC C165S mutant, purified as described previously (Nelson *et al.*, 2008; Poole and Ellis, 1996) and stored at −20 °C in 5 m*M* DTT. Prior to conducting experiments, DTT is removed using a Bio-Gel P6 spin column equilibrated in 25 m*M* potassium phosphate, pH 7.0, 100 μM DTPA.
3. *Escherichia coli R*-specific free methionine sulfoxide reductase (fRMsr) C84, 94S mutant, purified as described previously (Lin *et al.*, 2007), and stored at −80 °C in 5 m*M* DTT. Prior to conducting experiments, DTT is removed using a Bio-Gel P6 spin column equilibrated in 5 m*M* potassium phosphate, pH 7.0, 100 μM DTPA.
4. *E. coli* OxyR, "C4A-RD" with C208S mutation and C-terminal His-tag, expressed and purified as described in Section 3 and stored at −80 °C in 5 m*M* DTT. Prior to conducting experiments, DTT is removed using a Bio-Gel P6 spin column equilibrated in 50 m*M* Tris–HCl, pH 8.0, 100 μM DTPA.

3. Methods

3.1. Characterization of "DCP"-linked compounds

3.1.1. Specificity of DCP-linked probes for cysteine sulfenic acid

The first two fluorophore-linked probes generated from 1,3-cyclohexadione had in common the sulfenic acid-reactive 3-(2,4-dioxocyclohexyl) propyl (DCP) group to which the fluorophores were attached (Poole *et al.*, 2005). As all subsequent reagents also possess this reactive "core," we used the "DCP" abbreviation followed by the reporter designation to nickname all subsequent reagents (Poole *et al.*, 2007) (Fig. 3.2). All compounds were tested for their dimedone-like chemical properties using the sulfenic acid-containing C165S mutant of the bacterial peroxiredoxin AhpC (Ellis and Poole, 1997; Poole and Ellis, 2002) and measuring adduct formation by electrospray ionization mass spectrometry (ESI-MS). Using this approach, all compounds demonstrated reactivity with sulfenic acid similar to dimedone and gave distinct adducts with AhpC by mass spectrometry (Poole *et al.*, 2005, 2007). This result indicates that the addition of the hydrocarbon

chain and reporter group, and the lack of the dimethyl group present in dimedone, do not interfere with sulfenic acid reactivity. To confirm the specificity of these reagents and dimedone toward only the sulfenic acid forms of Cys, control reactions were conducted and demonstrated that the thiol, disulfide, or hyperoxidized forms of AhpC (wild type or C165S) did not react with the original two compounds (DCP-MAB and DCP-MCC) and dimedone, based on the lack of ESI-MS-detectable adduct formation (Poole *et al.*, 2005). To test for general cross-reactivity of these reagents with other oxidized sulfur-containing functional groups, we tested the reactivity of dimedone, as a model reagent, with one *S*-nitrosothiol and two sulfoxides. Dimedone did not react with *S*-nitrosoglutathione (GSNO) over 1 h at room temperature as judged by absorbance spectroscopy. In addition, nuclear magnetic resonance (NMR), spectroscopic, and chemical isolation experiments showed that dimedone does not react with aqueous solutions of either dimethyl sulfoxide or methionine sulfoxide. Although dimedone is known to react with both aldehydes and amines (Benitez and Allison, 1974; Halpern and James, 1964; Vogel, 2005), control reactions demonstrated that these reactivities are only exhibited under very basic or organic solvent conditions (Poole *et al.*, 2005). The failure of these same compounds to react with either reduced or oxidized wild type or reduced or hyperoxidized (sulfinic or sulfonic acids) C165S AhpC proteins also indicate that these compounds do not react with protein amine groups under these conditions. In addition, a C165S adduct with hydroxynonenal was unreactive with DCP-FL1. Taken together, these results demonstrate the specificity of the reaction of these compounds for sulfenic acids in proteins in aqueous buffers.

3.1.2. Measuring rates of DCP-linked probe incorporation into pure proteins

Reactivity of protein sulfenic acids toward dimedone-based chemical probes is a complex function of the accessibility, electrostatic microenvironment, and stability of the sulfenic acid species within each protein; the specific nature of the probe will undoubtedly influence the reaction rate as well. To measure the rate of reaction, the sulfenic acid (or potentially sulfenamide) form of pure proteins can be generated, incubated with the reagent of interest for varying times, and then rapidly exchanged via a Bio-Gel P6 spin column into ammonium bicarbonate for analysis by ESI-time of flight (TOF) or matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS. Changes in intensity of peaks corresponding to the various mass components observed can then be fit to an appropriate kinetic model to evaluate rates of alkylation by the reagent; this is best accomplished in cases where hyperoxidation of the sulfenic acid is relatively slow compared with alkylation.

Three proteins known to form regulatory or catalytic sulfenic acids were investigated to address probe reactivity using DCP-Bio1 (Fig. 3.2). The first two proteins, alkyl hydroperoxide reductase C component (AhpC) and a methionine sulfoxide reductase protein (fRMsr), are oxidative defense enzymes known to form a sulfenic acid intermediate at the active site Cys during the course of turnover with their respective substrates, hydroperoxides or *R*-methionine sulfoxide. For each protein, all Cys other than the peroxide-sensitive Cys were removed by mutagenesis (C165S mutant of AhpC, with Cys46 remaining, and C84,94S mutant of fRMsr, with Cys118 remaining) (Ellis and Poole, 1997; Lin *et al.*, 2007) in order to stabilize the active site sulfenic acid, at least with respect to disulfide bond formation which is normally the next step of the mechanism. Papain is a cysteine protease with a low pK_a Cys at the active site that is sensitive to oxidation by hydrogen peroxide, reversibly blocking its protease activity (Allison, 1976).

To assess sulfenic acid alkylation rates, proteins are first incubated with 10 m*M* DTT for 30 min at room temperature, then excess DTT is removed using a Bio-Gel P6 spin column preequilibrated in 25 m*M* potassium phosphate, pH 7.0, and 100 μM DTPA (DTPA is a metal chelator). At this point, stable sulfenic acid forms of the protein to be assayed can be generated in advance of the alkylation reaction, or the protein oxidation reaction can be conducted in the presence of the DCP-Bio1 to help promote alkylation and avoid hyperoxidation in the presence of excess oxidant or air. For the experiments to assess alkylation rates, the sulfenic acid form of fRMsr was prepared in advance by incubation with a 100-fold excess of methionine sulfoxide for 2 min and removal of the excess amino acid using a Bio-Gel P6 column, and then DCP-Bio1 was added. Because papain and C165S AhpC are somewhat prone to hyperoxidation under aerobic conditions, as noted during the MS analyses, these proteins were oxidized by one (AhpC) or two (papain) equivalents of hydrogen peroxide after the addition of DCP-Bio1. The reaction was allowed to proceed at pH 7.0 and, at various times, a portion of the reaction mixture was rapidly exchanged into 50 m*M* ammonium bicarbonate using a Bio-Gel P6 spin column and analyzed by MS (Table 3.1).

The rates of probe incorporation into the three proteins are very different, as shown in Table 3.1, with papain (1.65 min^{-1}) being faster than either fRMsr (0.13 min^{-1}) or AhpC (0.003 min^{-1}). These data suggest that the sulfenic acid intermediate in papain is more accessible and/or reactive than in C84, 94S fRMsr, and C165S AhpC. The results for AhpC are consistent with previous studies showing that alkylation of AhpC by IAAm is very slow, presumably due to relative inaccessibility of Cys46 at the active site (Nelson *et al.*, 2008).

Table 3.1 Rates of DCP-Bio1 incorporation into pure proteins at pH 7.0 and 25 °C[a]

Proteins	Rate (min^{-1})
Papain	1.65 ± 0.22
fRMsr	0.13 ± 0.014
AhpC	0.003 ± 0.0004

[a] For AhpC and papain, 50 μM of prereduced protein was incubated in the presence of 1 m*M* DCP-Bio1, 25 m*M* potassium phosphate, pH 7.0, 100 μM DTPA with one (AhpC) or two (papain) equivalents of hydrogen peroxide. fRMsr was oxidized with a 100-fold excess of methionine sulfoxide; excess methionine sulfoxide was removed using a Bio-Gel P6 column, and the protein was diluted into a solution containing 2 m*M* DCP-Bio1 to give final concentrations of 50 μM fRMsr and 1 m*M* DCP-Bio1 in 5 m*M* potassium phosphate buffer, pH 7.0. At each timepoint, the reaction was quenched by rapidly removing compound using a Bio-Gel P6 spin column equilibrated in 50 m*M* ammonium bicarbonate. For AhpC and papain, 50% acetonitrile and 1% formic acid were added to each sample followed by direct infusion into an ESI-TOF MS. fRMsr was measured by MALDI-TOF MS using sinapinic acid as the matrix. The time-dependent appearance of alkylated protein by MS analysis was fit to a single exponential equation to obtain first-order rates. Alternatively, both the oxidation and alkylation rates for papain could be evaluated using KinTekSim and the kinetic model A → B → C, where A is the R–SH form, B the R–SOH form, and C the biotinylated form of papain (Poole *et al.*, 2007).

3.1.3. Effects of pH on probe incorporation into pure proteins

Variation of buffer pH may affect the rate at which oxidized proteins are alkylated with the DCP-linked probes either due to a change in the inherent rate at which the probe reacts with sulfenic acids or due to a change in accessibility and/or microenvironment of the target sulfenic acid. To assess the effect of pH changes on reactivity of the sulfenic acid in fRMsr, oxidized protein was prepared as described above, then diluted 1:1 into buffers containing various concentrations of DCP-Bio1 to obtain final pH values of 5.5 and 8.0. First-order reaction rates from three independent experiments were obtained for each buffer and reagent concentration. Results with oxidized fRMsr indicated that the labeling rate for this protein is constant between pH 5.5 and 8.0 (an equivalent rate was also observed at pH 7, Table 3.1), with an overall second-order reaction rate of 0.12 ± 0.012 mM^{-1} min^{-1} (Fig. 3.3). These data suggest that there is no effect of pH between 5.5 and 8 on the inherent reactivity of DCP-Bio1 toward sulfenic acids.

3.2. Protocols for labeling cysteine sulfenic acids within cellular proteins

3.2.1. Choice of approaches for labeling cysteine sulfenic acids within cellular proteins

We have synthesized a range of sulfenic acid-directed compounds and the choice of compound will depend on the types of experiments that are planned. The biotin-linked compounds (DCP-Bio1, DCP-Bio2, and

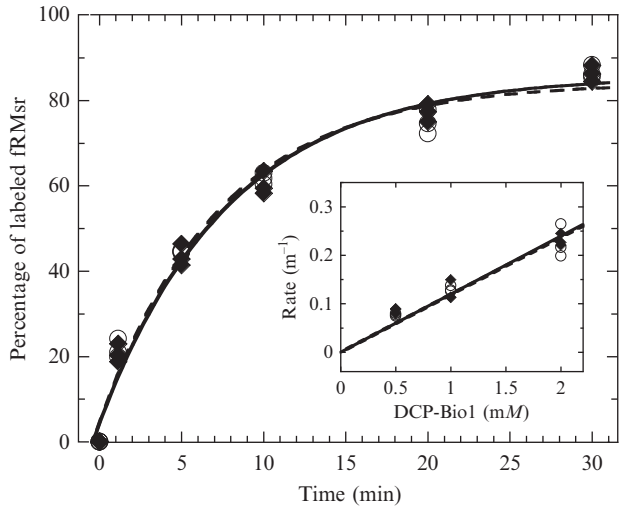

Figure 3.3 Effects of pH on incorporation of biotinylated probe into oxidized fMsr. Oxidized fRMsr (prepared by incubation with excess methionine sulfoxide in 5 mM potassium phosphate buffer, pH 7.0, as described in the text) was diluted 1:1 into either 50 mM MES, 100 mM DTPA, pH 5.3 or 50 mM Tris–HCl, 100 mM DTPA, pH 8.0, to a final concentration of 50 μM fRMsr, 0.5–2 mM DCP-Bio1, and a final pH of 5.5 (closed diamonds) or 8.0 (open circles). At the given incubation time, a sample of the reaction mixture was applied to a Bio-Gel P6 spin column to remove small molecules and exchange the protein into 50 mM ammonium bicarbonate, and then analyzed using MALDI-TOF MS using sinapinic acid as the matrix. Shown is the primary plot of the data obtained with 1 mM DCP-Bio, fit to a single exponential equation, yielding a first-order rate of 0.13 $\pm$ 0.014 min^{-1}. Using the secondary plot (inset), the second-order rates at both pH values were indistinguishable, at 0.12 $\pm$ 0.012 mM^{-1} min^{-1}. Each point represents a single replicate.

DCP-Bio3) are particularly powerful as they provide a means to affinity capture labeled proteins prior to analysis. DCP-Bio1 has been the most widely used among these reagents. We have also developed a series of compounds linked to fluorescent groups including methoxycoumarin (DCP-MCC), isatoic acid (DCP-MAB), fluorescein (DCP-FL1, DCP-FL2), and rhodamine (DCP-Rho1, DCP-Rho2). Finally, we have also generated an azide-linked reagent (DCP-N3) that can, after labeling, be further derivatized to any reporter group containing an alkyne or phosphine using either click chemistry or Staudinger ligation techniques, respectively (Reddie and Carroll, 2008).

With purified proteins, trapping of sulfenic acids can be conducted with reagent already present at the time of oxidant addition (AhpC and papain, above; Conway *et al.*, 2004), or with pretreatment of the protein prior to reagent addition, relying on generation of a relatively stable sulfenic acid

(fRMsr, above). Similarly, oxidized proteins can be cumulatively trapped over time within living cells or sampled at various times after treatment using lysis buffer containing the chemical probe. Neither approach is ideal; trapping of sulfenic acids at the time of cell lysis is dependent on the time chosen between stimulation and lysis and may cause certain sulfenic acids to be missed due to the transient nature of this species in many proteins. In contrast, trapping of sulfenic acids (e.g., by dimedone addition) within intact cells during the progression of signal transduction processes can and does alter the course and output of signaling pathways (Michalek *et al.*, 2007), and therefore does not reflect oxidation patterns of proteins during the normal course of signaling. Extent of labeling of given proteins using this latter approach may also reflect more of an accumulation of the product over time due to rapid redox cycling rather than serving as a readout of the amount of a given sulfenic acid form present at any one time in the intact cell. Because we are most interested in obtaining a ''snapshot'' of protein oxidation that reflects a given point in time after cell stimulation, we typically trap sulfenic acids during cell lysis. As there are situations where *in situ* labeling is more desirable, we provide below brief protocols for both approaches.

3.2.2. Protocol for "*in situ*" labeling of sulfenic acid-containing proteins in live cells

Cell permeability of the labeling reagent, which is observed with DCP-Bio1, DCP-Rho1, DCP-Rho2, and DCP-N3, allows for alkylation of protein sulfenic acids *in situ* prior to disruption of cells. Although one might expect the ester linkage of DCP-Bio1 to be subject to hydrolysis by nonspecific esterases in cells, our findings to date suggest that this reagent is resistant to such cleavage.

Briefly, cells of interest are grown in the appropriate media to 60–90% confluence in 100-mm dishes. The cells are then switched to media containing 100 μM DCP-Bio1 for a total of 30 or 60 min, and treated or not with the stimulant of interest during the course of this incubation. Following labeling, PBS is used to wash the cells three times to remove the excess DCP-Bio1 (or other reagent) and the stimulant. For further biochemical analyses, the cell lysates containing biotinylated proteins are analyzed using one of the methods described in the following chapter (Nelson *et al.*, 2010).

3.2.3. Protocol for labeling sulfenic acid-containing cellular proteins at time of lysis

Because cell lysates are exposed to oxidative stress as a result of lysis and exposure to atmospheric oxygen, the lysis buffer described here has been developed to minimize protein oxidation after cell disruption. Following treatment of cells with the stimulant of interest for the desired time, cells are washed with PBS to remove excess media and serum proteins, and

immediately scraped from the plate into lysis buffer containing DTPA, protease and phosphatase inhibitors, 1 m*M* DCP-Bio1, 200 units/ml catalase, 10 m*M* NEM, and 10 m*M* IAAm (note that phosphatase inhibitors may in some cases protect protein tyrosine phosphatases with oxidized Cys residues from being labeled by the probe). Typically, samples are incubated on ice for 1 h to let the reagent react with sulfenic acids then frozen at −80 °C to preserve the samples prior to analysis. We have found that sonication increases the amount of label incorporated into proteins, but may promote the adventitious oxidation that we are trying to avoid. In order to further protect against postlysis cysteine oxidation, we include catalase (which removes hydrogen peroxide) and DTPA (which complexes metals and prevents hydrogen peroxide generation through the Fenton reaction) to the lysis buffer containing the labeling agent. The NEM and IAAm are added to block free thiols and help prevent the formation of sulfenic acids after cell lysis. Previous studies have shown that individual Cys residues may be preferentially alkylated by either IAAm or NEM (Dennehy, 2006); therefore, we include both reagents. *Note*: for downstream MS analysis, it may be desirable to minimize the potential modifications and to only use one of the alkylating agents. Biotinylated proteins can be analyzed using methods shown in Figs. 3.4 and 3.5 and described in the accompanying chapter (Nelson *et al.*, 2010). As expected, excluding alkylating agents from the lysis buffer appears to cause an increase in nonspecific labeling of cellular proteins (Fig. 3.4), and this effect is further exacerbated if catalase is also excluded (not shown).

3.2.4. Effects of variables such as protein concentration and reagent concentration on extent of probe incorporation into cellular proteins

In addition to changes in lysis buffer components, the amount and/or concentration of cellular protein and the concentration of chemical trapping agent also affect the degree to which sulfenic acids are labeled. We have observed that the extent of DCP-Bio1 incorporation is affected by the protein concentration of the samples; as the protein concentration decreases, a higher percentage of cellular proteins are labeled by DCP-Bio1. This effect appears to be independent of stimulation with a cellular cytokine known to release intracellular reactive oxygen species, tumor necrosis factor α (Fig. 3.5A). The amount of label incorporation is also increased with increasing concentration of the DCP-Bio1 reagent, due at least in part to the better ability of the reagent to successfully outcompete other fates for the sulfenic acids (Fig. 3.5B). The presence of several strong bands in the samples, even in the absence of DCP-Bio1 (Fig. 3.5B), demonstrates the importance of including a "no reagent" control to identify protein bands that are present in the sample due to endogenous biotinylation. Together, these findings indicate that the optimal reagent

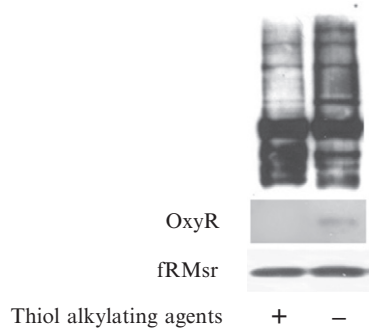

Figure 3.4 Addition of thiol alkylating agents helps block postlysis protein oxidation during incubation of cellular proteins with DCP-Bio1 in lysis buffer. For these experiments, HEK293 cells were grown in complete DMEM low glucose medium supplemented with 10% fetal bovine serum. Cells from each 100-mm plate were scraped into 1 ml PBS and transferred to microtubes. Cells were spun down and lysed with lysis buffer containing 0.1% SDS and protease and phosphatase inhibitors, as well as 1 m*M* DCP-Bio1 and 200 units/ml catalase. Thiol alkylating agents (10 m*M* NEM and 10 m*M* IAAm) were included or not as indicated. After incubation of the mixture on ice for 1 h, biotinylated proteins were captured using Streptavidin agarose resin. Mutant fRMsr was biotinylated with biotin maleimide (see Nelson *et al.*, 2010, for detailed protocol) and 1 μg fRMsr/500 μg of cell lysate was added to each sample prior to affinity capture for use as a procedural and loading control. Prereduced OxyR (mutated to contain only the peroxide-sensitive Cys) was included in the lysis buffer and used as a sensor of postlysis cysteine oxidation. The presence of biotinylated OxyR in the avidin-enriched material was visualized by Western blot using an antibody that recognizes the His tag.

concentration and cell number will have to be determined for each system and carefully matched in all experiments in order to obtain reproducible results.

3.2.5. OxyR as a reporter of postlysis cysteine oxidation

E. coli OxyR is a transcription factor which is directly activated by H_2O_2 through the oxidation of the reactive Cys residue, Cys199. In wild-type protein, the oxidation results in the formation of a sulfenic acid at Cys199 which subsequently reacts with Cys208 to form a disulfide bond (Choi *et al.*, 2001; Zheng *et al.*, 1998). A truncated construct of the C-terminal regulatory domain of OxyR lacking C208 as well as other nonperoxide sensitive cysteinyl residues, designated C4A-RD C208S OxyR, was previously generated (Choi *et al.*, 2001). Beginning with the pET21a-derived expression vector for this protein construct, we used the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) to remove the stop codon and express the protein with a C-terminal His tag.

The His-tagged C4A-RD C208S OxyR construct was expressed in *E. coli* strain B834 (DE3) using autoinduction medium PASM-5052

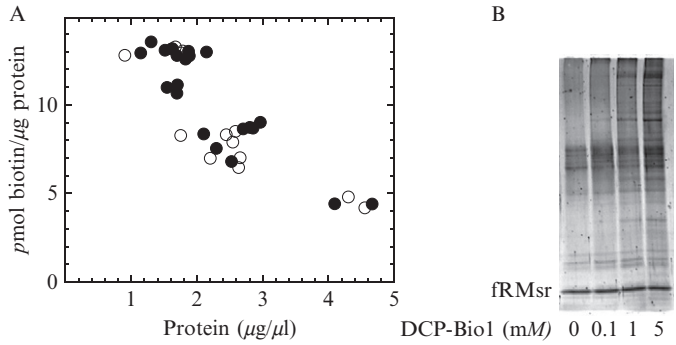

Figure 3.5 Effects of protein and reagent (DCP-Bio1) concentration on incorporation of biotin into cellular proteins. HEK293 cells were cultured and harvested as described in the text. After labeling with 1 m*M* DCP-Bio1, free probe was removed from the samples using a Bio-Gel P6 spin column and prepared for analyses as described in greater detail in the following chapter (Nelson *et al.*, 2010). In panel A, tumor necrosis factor alpha treated (closed circles) and untreated (open circles) samples were assessed for total biotin incorporation into proteins using the FluoReporter biotin incorporation assay kit from Invitrogen. For panel B, prebiotinylated fRMsr mutant was added to the starting protein concentrations prior to affinity capture for use as a procedural and loading control. Biotinylated proteins were captured using streptavidin-agarose, and extensively washed with 1% SDS, 4 *M* urea in PBS, 1 *M* NaCl, 100 m*M* ammonium bicarbonate, and deionized H_2O. The samples were eluted with 2% SDS in 50 m*M* Tris, pH 8.0, analyzed by SDS-PAGE, and stained with SYPRO Ruby.

overnight at 37 °C. Following centrifugation at 5000×*g* for 15 min, the washed cell pellets were resuspended in ~100 ml 50 m*M* sodium phosphate, pH 7.0, containing 10 m*M* 2-mercaptoethanol, and lysed with a pneumatic cell homogenizer (Avestin EmulsiFlex-C5). After centrifugation at 20,000×*g*, streptomycin sulfate (1%, w/v) was added to the supernatant, with stirring, for 15 min prior to centrifugation. The supernatant was filtered and bound to a Ni-NTA Superflow (Qiagen) column. The His-tagged C4A-RD C208S OxyR was eluted by gradually increasing the imidazole concentration to 250 m*M*. The eluted protein was concentrated and loaded onto a gel filtration (Superose 12 PG) column equilibrated with 50 m*M* Tris–HCl, pH 8.0, containing 100 m*M* NaCl, 100 μ*M* DTPA, and 2 m*M* DTT. The pure protein was concentrated to ~10 mg/ml based on an ε_{280} of 14,440 M^{-1} cm^{-1} and molecular weight of 26,470 Da (ε_{280} and molecular weight of His-tagged C4A-RD C208S OxyR were calculated using http://ca.expasy.org/tools/protparam.html) and stored at −80 °C.

Attempts to determine a rate for DCP-Bio1 incorporation into the OxyR construct as reported for the other three test proteins (Table 3.1) were inconclusive due to the high rate of hyperoxidation of this protein in the presence of oxygen or a second molecule of H_2O_2. With OxyR at

neutral pH, no further incorporation of DCP-Bio1 labeling is observed after 5 min and sulfinic and sulfonic acids can be observed within 2 min of the addition of 1.2 equivalents of H_2O_2. While this does not interfere with the ability to use OxyR as an effective sensor of adventitious oxidation occurring during lysis, it complicates the kinetic analyses.

As shown in Fig. 3.4, OxyR can be used as a "negative" control to monitor the amount of postlysis Cys oxidation. For this purpose, add reduced OxyR to the lysis buffer prior to harvesting the cells. The extent of undesired OxyR oxidation can be monitored by probing the OxyR content in the biotin pulldown using an antibody to the His-tag (Fig. 3.4). Using this technique, we confirmed that excluding thiol alkylating agents NEM and IAAm from the lysis buffer causes an increase in postlysis labeling (Fig. 3.4) since reduced OxyR becomes labeled under this experimental condition.

4. Summary

The development of a series of tagged sulfenic acid-directed compounds paves the way to determine the sites and proteins that are sensitive to cysteine oxidation in the cell as well as the cellular conditions under which such oxidations occur. We have shown that these compounds are reactive and specific. The rates with which DCP-Bio1 reacts toward sulfenic acids are significantly different for each of three pure proteins, papain, the C84, 94S mutant of fRMsr, and the C165S mutant of AhpC (Table 3.1), suggesting that the reaction of our DCP-linked probes is highly dependent on the accessibility and stability of sulfenic acid intermediates within their protein microenvironment. Interestingly, there was no difference in the rates of probe incorporation into fRMsr between pH 5.5 and 8.0. We have also provided protocols to label sulfenic acid modifications in cellular proteins; either *in situ* labeling of intact cells or labeling at the time of lysis can be conducted. We have investigated components of the lysis buffer and highly recommend the addition of alkylating reagents and catalase to prevent the formation of sulfenic acid subsequent to cell lysis. Data presented herein also indicate that the extent of labeling is highly dependent on protein concentration in the sample and highlight the need to standardize as much as possible the protein and reagent concentrations during labeling, especially when these reagents are applied to monitor temporal changes of oxidation or in comparative studies.

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REFERENCES

Allison, W. S. (1976). Formation and reactions of sulfenic acids in proteins. *Acc. Chem. Res.* **9,** 293–299.

Benitez, L. V., and Allison, W. S. (1974). The inactivation of the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase by dimedone and olefins. *J. Biol. Chem.* **249,** 6234–6243.

Charles, R. L., Schröder, E., May, G., Free, P., Gaffney, P. R., Wait, R., Begum, S., Heads, R. J., and Eaton, P. (2007). Protein sulfenation as a redox sensor: Proteomics studies using a novel biotinylated dimedone analogue. *Mol. Cell Proteomics* **6,** 1473–1484.

Choi, H., Kim, S., Mukhopadhyay, P., Cho, S., Woo, J., Storz, G., and Ryu, S. (2001). Structural basis of the redox switch in the OxyR transcription factor. *Cell* **105,** 103–113.

Conway, M. E., Poole, L. B., and Hutson, S. M. (2004). Roles for cysteine residues in the regulatory CXXC motif of human mitochondrial branched chain aminotransferase enzyme. *Biochemistry* **43,** 7356–7364.

Dennehy, M. K., Richards, K. A., Wernke, G. R., Shyr, Y., and Liebler, D. C. (2006). Cytosolic and nuclear protein targets of thiol-reactive electrophiles. *Chem. Res. Toxicol.* **19,** 20–29.

Ellis, H. R., and Poole, L. B. (1997). Roles for the two cysteine residues of AhpC in catalysis of peroxide reduction by alkyl hydroperoxide reductase from *Salmonella typhimurium*. *Biochemistry* **36,** 13349–13356.

Halpern, B., and James, L. B. (1964). Dimedone (5, 5-dimethylcyclohexane-1,3-dione) as a protecting agent for amino groups in peptide synthesis. *Aust. J. Chem.* **17,** 1282–1287.

Leonard, S. E., Reddie, K. G., and Carroll, K. S. (2009). Mining the thiol proteome for sulfenic acid modifications reveals new targets for oxidation in cells. *ACS Chem. Biol.* **4,** 783–799.

Lin, Z., Johnson, L. C., Weissbach, H., Brot, N., Lively, M. O., and Lowther, W. T. (2007). Free methionine-(R)-sulfoxide reductase from *Escherichia coli* reveals a new GAF domain function. *Proc. Natl. Acad. Sci. USA* **104,** 9597–9602.

Michalek, R. D., Nelson, K. J., Holbrook, B. C., Yi, J. S., Stridiron, D., Daniel, L. W., Fetrow, J. S., King, S. B., Poole, L. B., and Grayson, J. M. (2007). The requirement of reversible cysteine sulfenic acid formation for T cell activation and function. *J. Immunol.* **179,** 6456–6467.

Nelson, K. J., Parsonage, D., Hall, A., Karplus, P. A., and Poole, L. B. (2008). Cysteine pK_a values for the bacterial peroxiredoxin AhpC. *Biochemistry* **47,** 12860–12868.

Nelson, K. J., Klomsiri, C., Codreanu, S. G., Soito, L., Liebler, D. C., Rogers, L. A. C., Daniel, L. W., and Poole, L. B. (2010). Use of dimedone-based chemical probes for sulfenic acid detection: methods to visualize and identify labeled proteins. *Methods Enzymol.* **472,** 95–115.

Oshikawa, J., Urao, N., Kim, H. W., Kaplan, N., Razvi, M., McKinney, R., Poole, L. B., Fukai, T., and Ushio-Fukai, M. (2010). Extracellular SOD-derived H_2O_2 promotes VEGF signaling in caveolae/lipid rafts and post-ischemic angiogenesis in mice. *PLoS One* (in press).

Poole, L. B., and Ellis, H. R. (1996). Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 1. Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. *Biochemistry* **35,** 56–64.

Poole, L. B., and Ellis, H. R. (2002). Identification of cysteine sulfenic acid in AhpC of alkyl hydroperoxide reductase. *Methods Enzymol.* **348,** 122–136.

Poole, L. B., and Nelson, K. J. (2008). Discovering mechanisms of signaling-mediated cysteine oxidation. *Curr. Opin. Chem. Biol.* **12,** 18–24.

Poole, L. B., Karplus, P. A., and Claiborne, A. (2004). Protein sulfenic acids in redox signaling. *Annu. Rev. Pharmacol. Toxicol.* **44,** 325–347.

Poole, L. B., Zeng, B. B., Knaggs, S. A., Yakubu, M., and King, S. B. (2005). Synthesis of chemical probes to map sulfenic acid modifications on proteins. *Bioconjug. Chem.* **16,** 1624–1628.

Poole, L. B., Klomsiri, C., Knaggs, S. A., Furdui, C. M., Nelson, K. J., Thomas, M. J., Fetrow, J. S., Daniel, L. W., and King, S. B. (2007). Fluorescent and affinity-based tools to detect cysteine sulfenic acid formation in proteins. *Bioconjug. Chem.* **18,** 2004–2017.

Reddie, K. G., and Carroll, K. S. (2008). Expanding the functional diversity of proteins through cysteine oxidation. *Curr. Opin. Chem. Biol.* **12,** 746–754.

Reddie, K. G., Seo, Y. H., Muse Iii, W. B., Leonard, S. E., and Carroll, K. S. (2008). A chemical approach for detecting sulfenic acid-modified proteins in living cells. *Mol. Biosyst.* **4,** 521–531.

Salsbury, F. R., Jr., Knutson, S. T., Poole, L. B., and Fetrow, J. S. (2008). Functional site profiling and electrostatic analysis of cysteines modifiable to cysteine sulfenic acid. *Protein Sci.* **17,** 299–312.

Turell, L., Botti, H., Carballal, S., Ferrer-Sueta, G., Souza, J. M., Duran, R., Freeman, B. A., Radi, R., and Alvarez, B. (2008). Reactivity of sulfenic acid in human serum albumin. *Biochemistry* **47,** 358–367.

Vogel, A. I. (2005). Investigation and characterisation of organic compounds. *In* "Vogel's Textbook of Practical Organic Chemistry," (B. S. Furniss, ed.), pp. 1259–1260. Pearson, Singapore.

Zheng, M., Aslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279,** 1718–1721.