A Genetically Encoded Probe for Cysteine Sulfenic Acid Protein Modification in Vivo†

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ABSTRACT: All organisms have defense mechanisms to combat the deleterious effects of oxidative damage produced by reactive oxidative species (ROS). Although it is known that ROS play a major role in oxidative damage, increasing evidence reveals that ROS have wider cellular effects through their role in many signal transduction pathways. Here we have adapted a redox-regulated domain from the Yap1 transcription factor in *Saccharomyces cerevisiae* to function as a general trap for proteins that form cysteine sulfenic acid (Cys-SOH) in vivo. In response to H_2O_2, the Yap1 probe forms mixed disulfide bonds with a variety of proteins. The formation of these protein complexes is time dependent and peroxide concentration dependent. Disulfide-bonded complex formation can be attenuated by the addition of dimedone, a compound that specifically reacts with Cys-SOH, indicating the specificity of the probe toward Cys-SOH. An efficient one-step purification procedure was developed for proteins trapped by the Yap1 probe, and the constituents were identified by mass spectrometry. This methodology identified six proteins in *Escherichia coli* that contain redox-active cysteine residues known to form Cys-SOH as part of their catalytic cycle. The results suggest that the Yap1 probe is useful for identifying Cys-SOH-regulated proteins and can be employed in any genetically tractable organism to monitor transient Cys-SOH formation in vivo.

Oxidative stress involves a disturbance in the prooxidant−antioxidant balance in favor of prooxidants (*1*). Both endogenous and exogenous sources of reactive oxidative species (ROS) can contribute to oxidative stress and can include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and alkyl hydroperoxides (ROOH). If allowed to accumulate unchecked, these molecules exceed the normal antioxidant buffering capacity of the cell, leading to indiscriminate damage of cellular components including DNA, proteins, and lipids (*2*−*4*). In addition, recent studies have also implicated ROS, such as H_2O_2, in the regulation of specific signal transduction pathways. The goal of this work is to develop a genetically encoded probe for the discovery of novel proteins and signal transduction pathways that specifically perceive and respond to oxidative stress in living systems.

The major H_2O_2 response pathway in *Saccharomyces cerevisiae* involves the transcription factor Yap1 and the oxidant receptor protein Orp1 (*5*, *6*). This pathway is regulated by a novel disulfide bond-relay cascade to control the expression of ∼70 genes in response to H_2O_2 (*7*, *8*). Orp1 is homologous to glutathione peroxidase enzymes and has a biological function similar to that of the peroxiredoxin (Prx) protein family. It is responsible for H_2O_2 perception and subsequent catalysis of disulfide bond formation in Yap1 (*6*). The proposed Yap1 oxidation mechanism begins with the reaction of H_2O_2 with Cys36 in Orp1 and formation of a Cys36-SOH intermediate (*29*). Cys598 of Yap1 can then react with the Cys36-SOH intermediate to form a mixed-disulfide-bonded complex between Orp1 and Yap1 and ultimately results in the inhibition of a nuclear export sequence in Yap1 (*7*, *9*). An alternative pathway of H_2O_2-mediated redox regulation of Yap1 involves the thiol-specific antioxidant protein (Tsa1), which is also homologous to Prx proteins (*10*). Furthermore, in *Schizosaccharomyces pombe* it was demonstrated that the Yap1 homologue, Pap1, is also oxidized by a peroxiredoxin-like protein (Tpx1) in response to H_2O_2 (*11*). These examples of H_2O_2-mediated signal transduction all involve proteins that react with peroxides through thiol-based mechanisms, resulting in transient Cys-SOH[1] intermediates (Figure 1A). In each case, a specific cysteine (Cys598 in Yap1) located in the C-terminal cysteine-rich domain (cCRD) of Yap1/Pap1 reacts with the Cys-SOH intermediate, leading to the formation of a mixed disulfide bond (Figure 1B). The evidence that Cys598 in Yap1 can react with Cys-SOH intermediates on multiple nonhomologous Prx proteins suggests that the cCRD domain may generally react with Cys-SOH in any Prx-like protein. It also suggests that Yap1-cCRD could be used to capture Cys-SOH intermediates on non-Prx proteins as well.

The formation of Cys-SOH in proteins occurs through the reaction of cysteine thiols with H_2O_2 or alkyl hydroperoxides (*12*). Once formed, Cys-SOH intermediates are highly reactive and have proven difficult to identify and characterize in vivo. The chemical dimedone specifically reacts with the Cys-SOH and not the sulfhydryl form of cysteine (Figure 1A) (*13*). Dimedone has been used to capture stable Cys-

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[1] Abbreviations: CBB, Coomassie Brilliant Blue; Cys-SOH, cysteine sulfenic acid; Yap1-cCRD, Yap1 C-terminal cysteine-rich domain; CHP, cumene hydroperoxide; *t*-BOOH, *tert*-butyl hydroperoxide.

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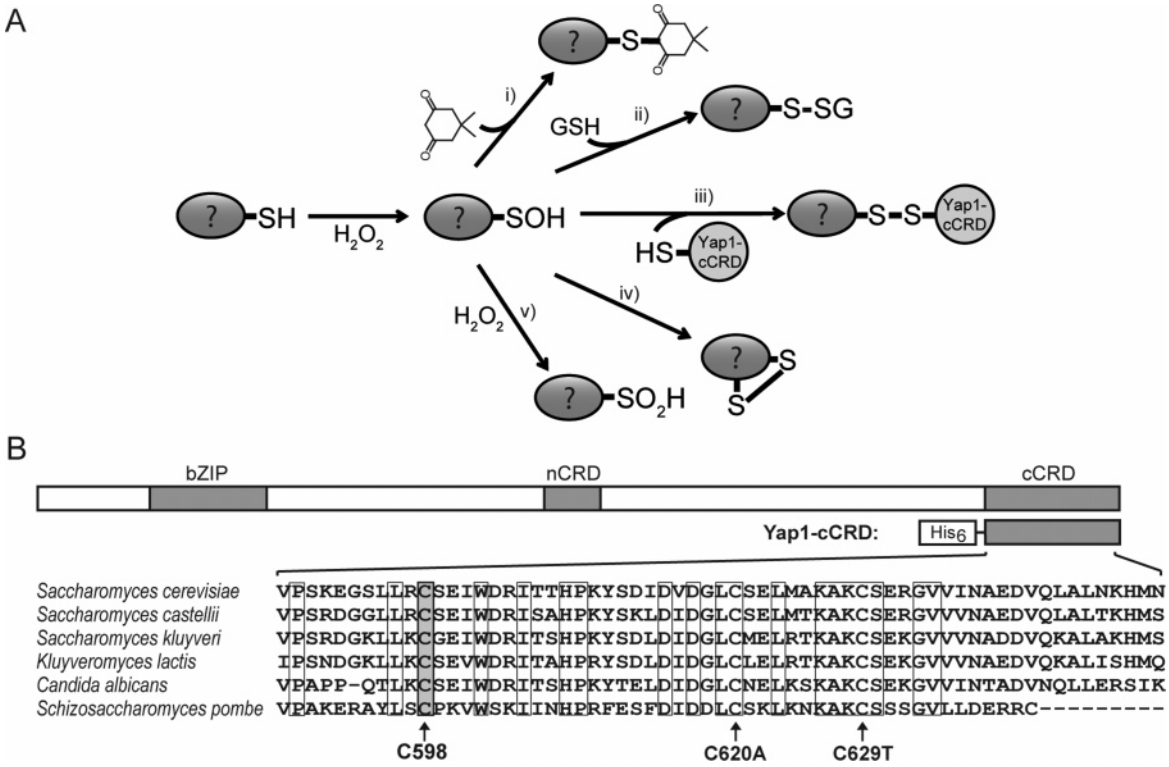

FIGURE 1: Protein Cys-SOH formation. **(**A**)** The reaction of H_2O_2 and low-pK_A cysteine residues results in the transient formation of Cys-SOH on proteins. This intermediate is very short-lived and further reacts to form a variety of end products. The chemical dimedone has been shown to react specifically with Cys-SOH and not the cysteine sulfhydryl (i) (*49*). If a second cysteine is in close proximity to the Cys-SOH, the sulfhydryl can react with the sulfur of the Cys-SOH, resulting in a disulfide bond (ii, iii, iv). In the case of Cys598 in Yap-cCRD, the reaction with Cys-SOH on a protein such as Orp1 results in a disulfide-linked complex (iii). If there are no other sulfhydryls present or a high concentration of oxidants, Cys-SOH can further react with the H_2O_2 to generate sulfinic acid (v). **(**B**)** The Yap1 protein contains a conserved bZIP domain and the N-terminal and C-terminal cysteine-rich domains (nCRD, cCRD). The Yap1-cCRD protein used for all experiments was comprised of the conserved cCRD, which included Asn565 to Asn650 with the Cys620 and Cys629 mutated to alanine and threonine, respectively. Cys598 is conserved in all Yap1 homologues as are many of the surrounding residues.

SOH protein modification in tissue extracts and has also been shown to react with AhpC in vitro upon exposure to oxidants (*14*, *15*). In vivo, Cys-SOH typically reacts with another cysteine, resulting in S-thiolation and disulfide bond formation (Figure 1A). This resolving cysteine can come from glutathione (GSH), a cysteine from the same protein, or a cysteine from a different protein. Cys-SOH can also be further oxidized to sulfinic acid (Cys-SO_2H), which can be reversed by the activity of sulfiredoxin proteins (*16*). Each of these S-thiolation reactions has the potential to regulate the biological function of a specific target protein (*9*, *11*, *17*−*19*). The extent to which Cys-SOH occurs in vivo is largely unknown because there are currently no methods for global proteomic-based Cys-SOH characterization in live cells or organisms. Here we report the development of Yap1--cCRD as a protein-based probe for capture of transient Cys-SOH formation on proteins in live organisms. This probe is genetically encoded and therefore can be used in any genetically tractable organism to capture proteins that undergo Cys-SOH modification.

MATERIALS AND METHODS

Yap1-cCRD Expression. A Yap1-cCRD expression construct was made for use in *Escherichia coli*. The Yap1 coding region corresponding to Asn565 to Asn650 was amplified by PCR. The PCR product was digested with *Bam*HI and *Not*I restriction enzymes and ligated into the pRSET vector in frame with an N-terminal His_6 tag. In addition, Cys620 and Cys629 were mutated to Ala and Thr, respectively; thus, Cys598 was the only cysteine in the expressed Yap1-cCRD protein. Yap1-cCRD was expressed in *E. coli* following the same protocols described previously for Orp1 and other His_6-tagged Yap1 proteins (*9*, *20*).

Protein Cys-SOH Capture in Vivo. The Yap1-cCRD expression construct in the pRSET vector containing an N-terminal His_6 tag was used for all Cys-SOH protein capture experiments. All Cys-SOH capture experiments were performed in the BL21(DE3) *pLysS* strain of *E. coli*. For each Cys-SOH capture experiment, a fresh single colony was used to inoculate 5 mL of Luria broth containing 100 μg/mL ampicillin and 40 μg/mL chloramphenicol. Cultures were grown overnight with shaking at 37 °C. The following day the cultures were diluted to an OD_{600} of 0.2 in fresh medium and grown to the mid-log phase. The expression of Yap1-cCRD was induced by addition of 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 20 min. The cultures were exposed to 0.5 mM H_2O_2, *tert*-butyl hydroperoxide (*t*-BOOH), or cumene hydroperoxide (CHP) for specified amounts of time. Disulfide-linked protein complexes were rapidly trapped by addition of 100% TCA directly to the cultures to a final concentration of 20% (w/v). The cells were pelleted by centrifugation and rinsed once in ice-cold 20% (w/v) TCA solution. The precipitated material was washed three times with acetone, and the remaining insoluble protein pellet was resuspended in 100 mM Tris (pH 8.0), 1% SDS, and 75 mM IAA and reacted in the dark at 37 °C for 30

min. The reactions were diluted 1:1 in H_2O, and the protein concentration of each extract was determined with the BCA protein concentration assay. Protein extracts were analyzed with reducing and nonreducing SDS−PAGE and transferred to nitrocellulose membranes, and Yap1-cCRD was visualized with horseradish peroxidase-linked anti-His_6 antibodies (Clontech). Immunoblots were visualized with a FujiFilm LSA-3000 imaging system.

Cys-SOH Protein Complex Purification and Identification. Disulfide-linked protein complexes containing Yap1-cCRD were selectively enriched by purification on a 5 mL Ni^{2+} affinity column (GE Healthcare). A 500 mL culture expressing Yap1-cCRD was exposed to 0.5 mM H_2O_2 for 2.5 min. TCA was added directly to the culture to a final concentration of 20% (w/v), and the cells were harvested as previously described. TCA-precipitated protein complexes were washed three times with acetone, and the pellets were air-dried for 5 min at 50 °C. Dried pellets were resuspended in buffer containing 100 mM Tris (pH 8.0), 1% SDS, and 75 mM IAA and reacted in the dark for 30 min at 37 °C. The insoluble proteins were removed with centrifugation, and the soluble fraction was dialyzed against a buffer comprised of 50 mM sodium phosphate (pH 8.0) and 300 mM NaCl. The dialyzed protein was diluted 1:1 with buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 30 mM imidazole, and 10% glycerol). The sample was applied to the Ni^{2+} affinity column and washed with 10 column volumes of buffer A. The column was loaded with 5 mL of buffer containing 5 mM DTT, removed from the FPLC system, and incubated for 2 h at 25 °C. After 2 h, the column was reattached to the system and DTT-sensitive proteins were eluted with 2 column volumes of buffer A. Two experiments were performed to control for nonspecific protein association with Yap1-cCRD. First, an equal-volume culture containing the Yap1-cCRD plasmid that was not treated with IPTG nor H_2O_2 was processed in parallel. Second, an equal-volume culture containing the Yap1-cCRD plasmid in which Cys598 was mutated to alanine was processed in parallel. The fractions eluted after DTT incubation were then concentrated to 250 μL with an Amicon centrifugal device and washed with 8 mL of buffer containing 50 mM sodium phosphate (pH 8.0) and 300 mM NaCl. Concentrated and washed protein samples were precipitated with methanol and chloroform and separated by reducing SDS−PAGE. Protein bands of interest were excised from the SDS−PAGE gel using a sterile blade.

The protein bands were submitted to the Genome Center Proteomics Core at the University of California, Davis, for mass spectrometry (LC−MS/MS) based protein identification. The proteins were reduced and alkylated according to previously described procedures (*21*) and digested with sequencing grade trypsin (Promega, Madison, WI). Protein identification was performed using an Eksigent Nano LC 2-D system (Eksigent, Dublin, CA) coupled to an LTQ ion trap mass spectrometer (Thermo-Fisher, San Jose, CA) through a New Objectives Picoview nanospray source. Peptides were loaded onto an Agilent nanotrap (Zorbax 300SB-C18, Agilent Technologies) at a loading flow rate of 5 μL/min. The peptides were then eluted from the trap and separated by a nanoscale 75 μm × 15 cm New Objectives picofrit column packed in house with Michrom Magic C18 AQ packing material. The peptides were eluted using a 40 min gradient of 2−80% buffer B (buffer A = 0.1% formic acid, buffer B = 95% acetonitrile/0.1% formic acid). The top 10 ions in each survey scan were subjected to automatic low-energy CID. Tandem mass spectra were extracted and charge state deconvoluted by BioWorks version 3.3. Deisotoping was not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, U.K.; version 2.1.03) and were searched against the entire *E. coli* K12 proteome database using the Global Proteome Machine Database (http://gpmdb.proteomics.ucdavis). Positive protein matches were ranked on the basis of their log(*e*) value, which corresponds to the expectation value of matching the protein randomly, and their log(*I*) score, which is a base 10 log of the sum of the intensities of the fragment ion spectra.

RESULTS

Development of Yap1-cCRD for Global Analysis of Cys-SOH Formation on Proteins in Vivo. The ability of Yap1-cCRD to form disulfide bonds with other proteins in response to oxidative stress was examined in vivo. Yap1-cCRD was overexpressed in *E. coli*, and these cultures were treated with 0.5 mM H_2O_2 for up to 10 min. Transiently formed disulfide-bonded proteins were trapped by the direct addition of TCA to the culture medium to reproducibly examine early time points after H_2O_2 exposure. Disulfide-bonded protein complexes involving Yap1-cCRD were observed on immunoblots using a monoclonal anti-His_6 antibody. The fully reduced Yap1-cCRD monomer migrated at ∼15 kDa, and the disulfide-bonded Yap1-cCRD dimer migrated at ∼32.5 kDa (Figure 2A). Under reducing conditions Yap1-cCRD migrated as a monomer, with a slight amount of unreduced dimer present. Under nonreducing conditions before H_2O_2 exposure, there were detectable disulfide-bonded complexes between Yap1-cCRD and unknown proteins (Figure 2A). Exposure to H_2O_2 resulted in an increase in the amount and abundance of Yap1-cCRD disulfide-linked complexes (Figure 2A). Up to 10 disulfide-bonded protein complexes involving Yap1-cCRD were resolved between 25 and 100 kDa. The levels of these complexes were maximal at 1 min after H_2O_2 exposure and were observed to decrease over a 10 min time course. As a control, the H_2O_2 exposure experiments were repeated with Yap1-cCRD, where Cys598 was mutated to alanine. As expected, for the Yap1-cCRD C598A mutant there were no disulfide-bonded protein complexes observed in response to H_2O_2 exposure (data not shown).

A consistently observed H_2O_2-inducible band was detected on the immunoblot at ∼62 kDa. We identified the components of this protein complex and used this 62 kDa band as an indicator for Yap1-cCRD disulfide-linked complex formation in other Cys-SOH trapping experiments. We treated a 500 mL culture with H_2O_2 and prepared a TCA-trapped protein extract. This extract was purified over a Ni^{2+} affinity column, and the imidazole-eluted fraction was concentrated and analyzed with SDS−PAGE. Immunoblot analysis showed the presence of a 62 kDa band that migrated at the same apparent molecular weight as the complex observed in crude protein extracts (Figure 2B). This band disappeared under reducing conditions, indicating that it was comprised of a disulfide bond between Yap1-cCRD and an unknown protein. This band was not present in a control sample that was not induced with IPTG nor treated with H_2O_2. The 62 kDa band

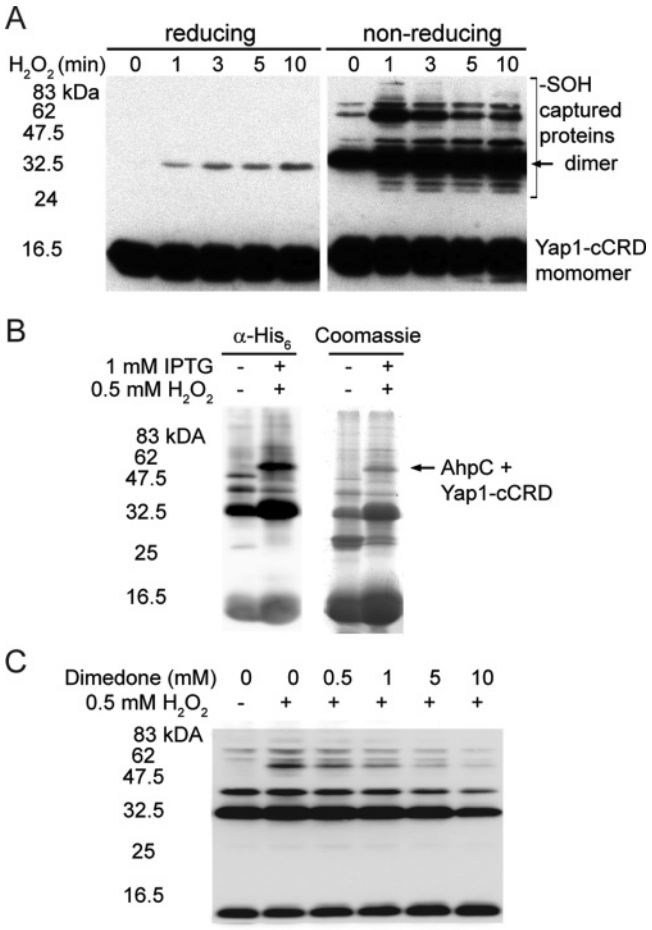

FIGURE 2: Yap1-cCRD probe for Cys-SOH proteins in vivo. **(A)** Protein extracts from *E. coli* cultures expressing Yap1-cCRD were used to visualize the formation of H_2O_2-induced protein complexes. TCA-prepared protein extracts were separated with reducing and nonreducing SDS−PAGE and probed with anti-His_6 antibodies. The Yap1-cCRD monomer is observed at ∼15 kDa, and a disulfide-bonded Yap1-cCRD dimer can be observed at ∼32.5 kDa under nonreducing conditions. Upon exposure to H_2O_2, the formation of higher molecular weight protein complexes involving Yap1-cCRD is observed. When the protein extracts are separated under reducing conditions, few of these protein complexes are observed, indicating the presence of disulfide bonds. **(B)** Large-scale Ni^{2+} affinity chromatography purification of protein extracts that were untreated or treated with H_2O_2. The protein complexes were eluted with imidazole, concentrated, and separated with nonreducing SDS−PAGE. The gels were transferred to nitrocellulose membranes for immunoblotting; the total protein content was analyzed with CBB stain. There is a distinct H_2O_2-inducible band at ∼62 kDa observed in the immunoblot and CBB-stained gel. LC−MS/MS determined that both Yap-cCRD and AhpC were identified in this band. **(C)** *E. coli* cultures expressing Yap1-cCRD were pretreated with increasing concentrations of dimedone, which is a compound that specifically reacts with Cys-SOH. These cultures were exposed to H_2O_2 for 1 min, and TCA-trapped protein extracts were prepared and separated by nonreducing SDS−PAGE. The gels were transferred to nitrocellulose membranes and probed with an anti-His_6 antibody. Dimedone pretreatment attenuates H_2O_2-induced Yap1-cCRD protein complex formation as indicated by dose-dependent decreases in the quantity of disulfide-bonded complexes.

was also visible when the gel was stained with Coomassie Brilliant Blue (CBB) (Figure 2B). The protein component of this band was identified with in-gel proteolysis and LC−MS/MS and was shown to contain alkyl hydroperoxide reductase C (AhpC), a well-characterized peroxiredoxin with a molecular weight of 20 700. Purified AhpC forms Cys-SOH upon treatment with H_2O_2 (*22*). These data provide support that Yap1-cCRD reacts with Cys-SOH-containing proteins in vivo in response to H_2O_2. Furthermore, it suggests that the AhpC:Yap1-cCRD band can be used as a reliable indicator for the capture of Cys-SOH in our system.

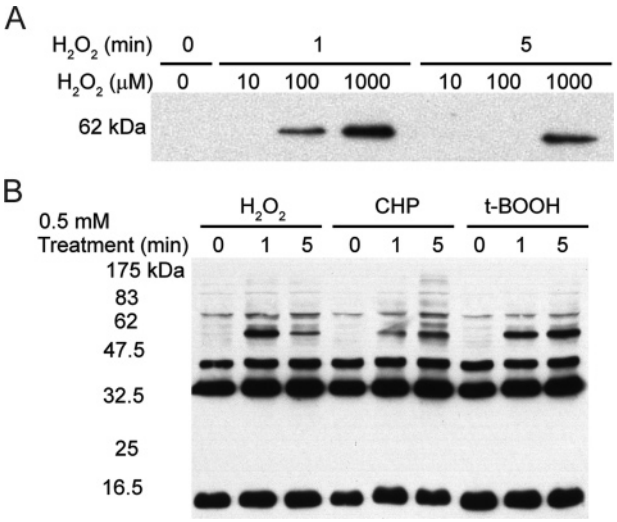

FIGURE 3: Effects of H_2O_2 concentration and peroxides on Yap1-cCRD complex formation. **(A)** *E. coli* was treated with 0, 10, 100, and 1000 μM H_2O_2 for 0, 1, and 5 min. After treatment with 100 μM H_2O_2, the protein complex formation peaks at 1 min and is undetectable after 5 min. A higher level of complex formation can be seen after treatment with 1000 μM H_2O_2 that also peaks at 1 min and decreases after 5 min. **(B)** The effects of CHP and *t*-BOOH were compared to the effects of H_2O_2 on *E. coli* expressing Yap1-cCRD. A time course was taken at 0, 1, and 5 min for H_2O_2, CHP, and *t*-BOOH.

To confirm our initial results, we examined the role of Cys-SOH in protein complex formation by pretreatment of *E. coli* cultures with dimedone. The cultures were pretreated with 0.5, 1, 5, and 10 mM dimedone for 30 min prior to exposure to 0.5 mM H_2O_2. As a control a culture was also pretreated with DMSO, the vehicle for dimedone. Analysis of the 62 kDa AhpC:Yap1-cCRD band showed that it was induced upon treatment with H_2O_2 (Figure 2C). At dosages of 5 and 10 mM dimedone pretreatment, the intensity of the AhpC:Yap1-cCRD band was similar to that of the untreated sample that was not induced with H_2O_2 (Figure 2C). Furthermore, we observed similar decreases in intensity of many of the Yap1-cCRD-trapped proteins upon pretreatment with dimedone. These data further suggest that the Yap1-cCRD disulfide-bonded complexes are formed through the specific reaction of Cys598 with Cys-SOH on multiple proteins and that pretreatment with a compound that competitively reacts with Cys-SOH attenuates disulfide-linked complex formation.

Influence of the Oxidant Level and Type on Yap1-cCRD Cys-SOH Capture. We wanted to determine the sensitivity of disulfide bond complex formation to varying levels of H_2O_2 exposure. Cultures were treated with 0, 10, 100, and 1000 μM H_2O_2 for 0, 1, and 5 min. Exposure to 10 μM H_2O_2 did not result in any detectable Yap1-cCRD protein complex formation (Figure 3A). However, exposure to 100 μM H_2O_2 for 1 min resulted in the formation of the AhpC:Yap1-cCRD protein complex. The amount of this protein complex increased upon exposure to 1000 μM H_2O_2. The amounts of the AhpC:Yap1-cCRD complex were maximal at 1 min for

all samples. For the culture exposed to 100 μM H_2O_2, the AhpC:Yap1-cCRD complex was gone at 5 min. Additionally, we wanted to test whether Yap1-cCRD could capture Cys-SOH on proteins after exposure to *t*-BOOH and CHP. We hypothesized that because both of these peroxides are more lipid soluble than H_2O_2, they could react with a different subset of target proteins. Upon exposure to *t*-BOOH and CHP we observed the induction of Yap1-cCRD disulfide-bonded complexes similar to those observed upon H_2O_2 exposure (Figure 3B). The CHP exposure resulted in more Yap1-cCRD disulfide-bonded complexes than either H_2O_2 or *t*-BOOH exposure. CHP exposure also resulted in reaction kinetics different from that of H_2O_2 exposure. For CHP exposure, protein complex formation was maximal at 5 min, compared to 1 min for H_2O_2 exposure. *t*-BOOH exposure also resulted in increased protein complex formation at 5 min compared to the kinetics of H_2O_2 exposure, but did not result in the delay observed with CHP. These differences may result from longer half-lives of CHP and *t*-BOOH in vivo or slower permeability of each compound through the outer and inner membranes of *E. coli*. Further proteomic analyses of the protein complexes formed with Yap1-cCRD in response to these various oxidants will indicate differences in their protein target preferences.

Purification and Identification of Yap1-cCRD Disulfide-Linked Protein Complexes. To further confirm that Yap1-cCRD was able to capture Cys-SOH formation on proteins upon oxidant exposure, we performed a large-scale preparation and purification of these disulfide-linked complexes (Figure 4A). As a negative control for nonspecific binding, we also prepared large-scale protein extracts from cultures that were not induced with IPTG nor exposed to H_2O_2. Affinity-purified samples were separated with reducing SDS−PAGE (Figure 4B). The negative control sample showed no detectable protein bands with CBB stain. In the sample that had been exposed to H_2O_2 there were five protein bands of strong intensity and greater than 10 protein bands of weak intensity (Figure 4B). The Yap1-cCRD protein was not observed in the DTT-eluted fraction, and neither were proteins known to nonspecifically bind Ni^{2+} affinity columns. These results demonstrate that this method allows for selective enrichment of trapped Yap1-cCRD disulfide-bonded complexes for proteomic analysis.

We used in-gel proteolysis and LC−MS/MS to identify the proteins that form H_2O_2-inducible disulfide bonds with Yap1-cCRD. The four strong-intensity bands were analyzed individually, and the remainder of the gel was subsectioned into seven areas for analysis (Figure 4B). Table 1 lists the positive protein matches for each of the strong protein bands, and the identifications are also listed next to the gel. Of the five most intense protein bands, three corresponded to known Prx-like proteins that form Cys-SOH in response to peroxides (*23*, *24*). Tpx and AhpC are known to form Cys-SOH in vitro in response to peroxides. BCP is also likely to form Cys-SOH as part of its catalytic cycle on the basis of its homology to the Prx protein family. These three proteins are the only Prx-like proteins in *E. coli*. The ~83 kDa band was identified as DnaK and was also observed in control experiments in which Cys598 of Yap1-cCRD was mutated to alanine, indicating that it nonspecifically associates with Yap1-cCRD (data not shown). In light of this observation, chaperone proteins captured by the Yap1-cCRD probe could

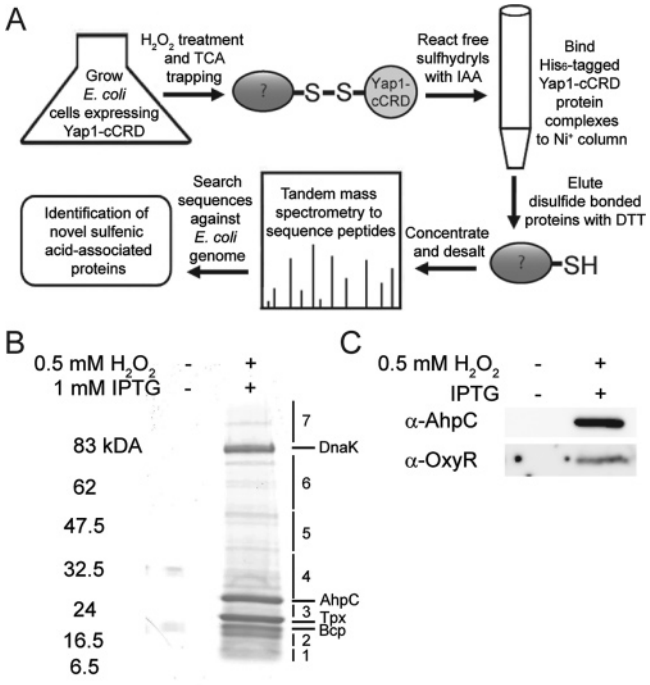

FIGURE 4: Purification and identification of H_2O_2-inducible Yap1-cCRD protein complexes. **(**A**)** Schematic representation of the methodology used to purify and identify trapped proteins with LC−MS/MS. *E. coli* cultures expressing Yap1-cCRD were treated with H_2O_2. These cultures were trapped and extracted in the presence of TCA, free sulfhydryls were reacted with IAA, and the extracts were applied to Ni^{2+} affinity columns. Proteins that were disulfide linked to Yap1-cCRD were eluted from the column by incubation with DTT for 2 h at 25 °C. Eluted proteins were concentrated with an Amicon filter and desalted by methanol−chloroform precipitation. The precipitated proteins were then either digested with trypsin and analyzed with LC−MS/MS or separated by SDS−PAGE and then analyzed with LC−MS/MS. The LC−MS/MS data were searched against an *E. coli* proteomic database to identify the Yap1-cCRD disulfide-linked proteins. **(**B**)** SDS−PAGE analysis of DTT-eluted Yap1-cCRD disulfide-linked proteins stained with CBB stain. The control sample was not treated with IPTG nor H_2O_2. We analyzed a control sample in which Cys598 of Yap1-cCRD was mutated to alanine, which showed similar results. In the H_2O_2-induced samples, there are four high-intensity protein bands that were identified as Bcp, Tpx, AhpC, and DnaK. DnaK was also observed in control experiments in which Cys598 of Yap1-cCRD was mutated to alanine, indicating that it nonspecifically associates with Yap1-cCRD. Seven regions of the gels were also subjected to LC−MS/MS, and the identified high-abundance proteins are listed in Table 1. **(**C**)** The same DTT elution fractions as shown in (B) were separated with SDS−PAGE, transferred to nitrocellulose membranes, and probed with anti-AhpC or anti-OxyR. Both AhpC and OxyR were observed in the H_2O_2-induced samples, but not observed in the control samples.

be the result of noncovalent protein−protein interactions. The lowest molecular weight band appeared to be a mixture of TrxA, TusE, PpiC, IscA, and SufA proteins. Of these, only PpiC, which is part of the peptidylprolyl isomerase family, does not have a biological function that involves redox-active cysteine residues. Table 1 also lists the most abundant, positively identified proteins found in the subsections of the gel. We identified three proteins in which Cys-SOH is part of the catalytic mechanism. Both methionine sulfoxide reductase (Msr) A and B have been previously shown to have a sulfenic acid intermediate in their catalytic cycle (*25*, *26*). BtuE, which is homologous to the Orp1 family of peroxidases, was also identified as a protein that forms Cys-SOH as part of its catalytic cycle (Supporting Information

Table 1: Proteins Identified That Form H_2O_2-Inducible Disulfide Bonds with Yap1-cCRD

gel section	protein	description	log(*l*)	no. of Cys residues	MW	redox-active Cys	Cys-SOH	ref
	Bcp	peroxiredoxin	6.74	3	17 600	yes	yes	*38*
	Tpx	peroxiredoxin	7.92	3	17 800	yes	yes	*39*
	AhpC	peroxiredoxin	7.79	2	20 700	yes	yes	*22*
	DnaK	chaperone	6.62	1	69 100			
1	TrxA	oxidoreductase	5.74	2	11 800	yes		*40*
1	TusE	sulfite reductase	5.58	1	12 400	yes		*41*
1	PpiC	prolyl isomerase	5.41	2	10 200			
1	IscA	Fe−S assembly	5.3	3	11 500	yes		*42*
1	SufA	Fe−S assembly	5.1	3	12 100	yes		*43*
2	IscU	Fe−S assembly	5.11	3	13 800	yes		*44*
2	50S L11	peptide synthesis	5.08	1	14 900			
2	HNS	transcriptional regulation	4.97	1	15 500			
2	MsrB	oxidative stress response	4.81	6	15 400	yes	yes	*26*
3	peptide deformylase	protein synthesis	5.43	2	19 300			
3	NuoE	NADH dehydrogenase	6.1	5	18 600	yes		*45*
3	50S L6	peptide synthesis	5.9	1	18 900			
3	BtuE	GSH/thiol peroxidase	5.48	3	20 500	yes	yes	
4	gntY	oxidoreductase, Trx-like protein	4.93	4	21 000			
4	Adk	purine ribonucleotide biosynthesis	4.76	1	23 600			
4	ybiS	uncharacterized	4.54	2	33 300			
4	MsrA	oxidative stress response	3.17	4	23 300	yes	yes	*25*
5	RpoA	RNA synthesis	6	4	36 500			
5	Tsf	translation elongation	5.81	2	30 400			
5	YgfZ	putative global regulator	5.21	2	36 100			
5	Icd	TCA cycle	5.15	6	45 700			
5	Hsp33	oxidative stress/chaperone	5.06	6	32 500	yes		*27*
6	TufB	translation elongation	6.16	3	43 300			
6	30S S1	protein synthesis	6.65	2	61 100			
6	NusA	translation elongation	6.32	3	54 800			
7	ClpB	protein degradation	5.46	3	95 500			
7	AceF	dihydrolipoamide dehydrogenase	4.53	1	66 100	yes		*46*
7	ThiI	sulfur transferase	4.11	5	54 900	yes		*47, 48*

Figure 1). Of the remaining low-intensity proteins identified, many are known to be involved in redox processes and to use cysteine or sulfur as part of their biological function. One example is the redox-regulated molecular chaperone, Hsp33, which forms two disulfide bonds in response to oxidative stress (*27*). Further examples are the IscA, SufA, and IscU proteins that contain conserved redox-active cysteine residues that are involved in Fe−S cluster biosynthesis (*28*). The proteins identified in Table 1 provide support that this methodology can be used to capture and enrich for Cys-SOH-containing proteins. However, it may be that the Yap1-cCRD probe also captures proteins that contain highly reactive cysteine residues, with lowered sulfhydryl pK_A values, in addition to capture of proteins that form Cys-SOH. Interestingly, our recent investigation into the molecular mechanisms of H_2O_2 reactivity of the Orp1 protein showed that a low-pK_A cysteine residue was responsible for H_2O_2-induced Cys-SOH formation (*29*). Therefore, the confirmation of protein Cys-SOH modification and sulfhydryl pK_A should be verified with conventional biochemical methodologies (*23*, *29*).

To confirm the presence of specific proteins in the H_2O_2-exposed samples that are known to form Cys-SOH in response to H_2O_2, we performed immunoblots using antibodies specific for AhpC and the OxyR transcription factor (*22*, *30*). Both AhpC and OxyR were observed in the samples that had been exposed to H_2O_2, but were not observed in the negative control samples (Figure 4C). While AhpC could be clearly observed on CBB-stained gels and identified with LC−MS/MS, OxyR could not. This is most likely because of the transient nature of Cys-SOH formation in OxyR and the low levels of OxyR in the cell (*30*). It is likely that if the sampling size were to be increased, OxyR could be positively identified using this methodology. However, the identification of low-abundance proteins that form Cys-SOH remains challenging given that they could be masked by more abundant Cys-SOH proteins, even after enrichment with affinity chromatography.

DISCUSSION

An important conclusion from this study is that Yap1-cCRD can function as a Cys-SOH probe when expressed in non-native organisms. It is able to transiently capture potentially novel Cys-SOH modifications on proteins in real time. This underscores the potential application of Yap1-cCRD as a general probe for Cys-SOH formation in proteins. Our data suggest that Yap1-cCRD will be useful in uncovering redox-regulated pathways that involve Cys-SOH intermediates in any genetically tractable organism or cell culture. We do not anticipate problems with heterologous expression in other organisms since the Yap1-cCRD sequence is not conserved in higher eukaryotes and should not interfere with endogenous signaling pathways. The Yap1-cCRD sequence can be functionalized with multiple epitope or purification tags. One limitation of this approach is that the acidic conditions required for trapping of disulfide bonds may preclude the use of affinity tags that require a folded structure. The use of metal affinity chromatography results in minimal background interference and is suited for purification under a variety of stringent conditions. Reduction of the disulfide bonds between Yap1-cCRD and the Cys-SOH-captured proteins by DTT incubation eliminated back-

ground proteins that were nonspecifically bound to the Ni^{2+} column as well as contamination of large amounts of Yap1-cCRD, which remain bound to the column.

Multiple proteomic-based techniques have emerged for the global analysis of protein redox regulation (*31*−*33*). These techniques are based on the differential reactivity of reduced and oxidized sulfhydryl groups toward electrophilic compounds in cell extracts and have been used successfully to monitor thiol oxidation in microorganisms, plants, cultured cells, and tissues. Eaton and colleagues have developed methodologies for specifically monitoring Cys-SOH formation in proteins both in cellular extracts and in situ in isolated rat hearts (*14*, *34*). The first method utilized an arsenite reduction step, which specifically reduced the Cys-SOH back to its sulfhydryl form and thereby allowed for the detection of sulfhydryl in proteins that formed a stable Cys-SOH modification (*14*). More recently they have developed a biotinylated dimedone analogue that can be used to capture Cys-SOH formation on proteins in both cellular extracts and perfused rat hearts (*34*). Using this novel chemistry, they were able to identify >20 proteins that form Cys-SOH upon exposure to H_2O_2. Comparison of the proteins identified with dimedone and Yap1-cCRD capture shows no similar Cys-SOH-modified proteins. This is not surprising, since the studies were conducted in different organisms using completely different chemical approaches. The difference in protein specificity could also be explained by differences in the reaction rates of the two probes for Cys-SOH. It would be useful to compare the two approaches in a common organism, under similar conditions. Such a study could be performed in *E. coli* or any other genetically tractable organism. We anticipate that the two methodologies will be complementary and that perhaps each could be used to identify different subsets of Cys-SOH-modified proteins.

The selective capture of redox-regulated proteins in living systems is not without precedent (*35*). One example of selective capture of redox-regulated proteins involved the use of a mutant form of the DsbA protein oxidoreductase from *E. coli* containing a His_{10} tag (*36*). Mutation of a conserved proline residue to threonine resulted in the trapping of DsbA target proteins via a mixed disulfide bond. The trapped protein complexes were then affinity purified, and the protein substrates of DsbA were identified with gel electrophoresis and LC−MS/MS. Methodologies involving capture of redox-active proteins in vivo with an affinity-tagged protein, such as Yap1-cCRD or DsbA, can be limited by the reductive pathways in the cell. Since the capture of unknown proteins involves disulfide bonds, the complexes may be only transiently stable in vivo. In the case of Yap1-cCRD, we observed maximal induction of complex formation immediately after H_2O_2 exposure and reduction of these disulfide-linked complexes over a 10 min time span. This demonstrates the need for rapid analysis and processing of the samples and, potentially, the use of strains that contain mutations in the glutathione or thioredoxin NADPH-based reduction pathways. However, final confirmation of Cys-SOH formation on protein identified with this methodology requires analysis of the protein in a purified system with mass spectrometry, (NBD)Cl modification, or dimedone modification.

We were able to use Yap1-cCRD to monitor Cys-SOH formation in a non-native organism, which highlights the ubiquitous reactivity of the Yap1-cCRD Cys598 residue. In both *S. cerevisiae* and *S. pombe*, peroxiredoxin-like enzymes have been shown to regulate the Yap1 and Pap1 transcription factors (*11*, *37*). In each case, disulfide-linked complexes between Tsa1 and Yap1 or Tpx1 and Pap1 were not observed. Our data also suggest that other cysteine-containing proteins may form Cys-SOH in response to H_2O_2 and alkyl hydroperoxide exposure (Table 1). Our results represent progress in the identification of Cys-SOH posttranslational modifications in vivo. In the case of peroxiredoxins, the Cys-SOH intermediate is short-lived, thus making it difficult to capture. In redox proteomic studies in *E. coli*, Tpx was also identified as a redox-sensitive protein (*33*). Using our method, we were able to capture low-abundance proteins that form Cys-SOH, including the OxyR transcription factor. Overall, this work adds a genetically encoded tool to the emerging field of redox proteomics that is useful in investigating redox regulation of protein function in vivo.

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SUPPORTING INFORMATION AVAILABLE

Additional details about protein sequences and a sequence alignment of BtuE and glutathione peroxidases from *S. cerevisiae* (Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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