Keap1, the Sensor for Electrophiles and Oxidants that Regulates the Phase 2 Response, Is a Zinc Metalloprotein †

ARTICLE *in* BIOCHEMISTRY · JUNE 2005

Impact Factor: 3.02 · DOI: 10.1021/bi047434h · Source: PubMed

3 AUTHORS, INCLUDING:

Albena T Dinkova-Kostova
University of Dundee
106 PUBLICATIONS **8,019** CITATIONS

SEE PROFILE

Nobunao Wakabayashi
University of Pittsburgh
52 PUBLICATIONS **8,342** CITATIONS

SEE PROFILE

Keap1, the Sensor for Electrophiles and Oxidants that Regulates the Phase 2 Response, Is a Zinc Metalloprotein[†]

Albena T. Dinkova-Kostova,* W. David Holtzclaw, and Nobunao Wakabayashi

The Lewis B. and Dorothy Cullman Cancer Chemoprotection Center, The Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received December 7, 2004; Revised Manuscript Received March 8, 2005

ABSTRACT: Induction of the phase 2 response, a major cellular reaction to oxidative/electrophile stress depends on a protein triad: actin-tethered Keap1 that binds to Nrf2. Inducers react with Keap1 releasing Nrf2 for nuclear translocation and activation of the antioxidant response element (ARE), which regulates phase 2 genes. The primary sensors for inducers are certain uniquely reactive cysteine thiols of Keap1. Recombinant murine Keap1 contains 0.9 zinc atoms per monomer as determined by inductively coupled plasma-optical emission spectrometry: its zinc content depends on the metal composition of the overexpression medium. Simultaneous direct measurement of bound zinc using a pyridazoresorcinol chelator and protein thiol groups using 4,4′-dipyridyl disulfide has established that (i) zinc is bound to reactive cysteine thiols of Keap1 and is displaced stoichiometrically by inducers, (ii) with these cysteines mutated to alanine, the affinity for zinc is reduced by nearly 2 orders of magnitude, and (iii) the association constant of Keap1 for zinc is 1.02 ($\pm$0.19) $\times$ 10^{11} M^{-1}, consistent with a Zn^{2+} metalloprotein. Co^{2+} substitution for Zn^{2+} yields an optical spectrum consistent with tetrahedral metal coordination. Coincident binding of inducers and release of zinc alters the conformation of Keap1, as shown by a profound decline of its tryptophan fluorescence and depression of fluorescence of a hydrophobicity probe. Thus, regulation of the phase 2 response involves chemical modification of critical cysteine residues of Keap1, whose reactivity is modulated by zinc binding. Keap1 is a zinc-thiol protein endowed with a delicate switch controlled by both metal-binding and thiol reactivity.

Aerobic cells counterattack electrophile and oxidant stresses by a variety of cytoprotective (phase 2) proteins, many of which are enzymes and include glutathione transferases (GST), NAD(P)H:quinone oxidoreductases (NQO1), and heme oxygenase 1 (HO-1). Under basal conditions, these protective enzymes are present at a fraction of their full capacity, but transcription of their cognate genes can be coordinately upregulated by exposure to a variety of chemical agents, thereby providing enhanced protection against carcinogens, mutagens, and other forms of oxidant and electrophile toxicities (see reviews *1*−*3*). Because a number of potent inducers of these cytoprotective proteins are found in edible plants (e.g., sulforaphane in crucifers), the prospect of dietary protection against disease appears very attractive.

Three cellular components are of central importance for the mechanisms by which the levels of phase 2 proteins are regulated: (i) antioxidant response elements (ARE),[1] enhancers of transcription that are present in the upstream regions of several (perhaps all) phase 2 genes and have the consensus sequence: TGAG/CNNNGC (*4*); (ii) Nrf2 (nuclear factor-erythroid 2-related factor 2), a basic leucine zipper transcription factor of the "cap-'n collar" family that is itself inducible and binds in heterodimeric combination with other factors, such as small Maf, to ARE enhancers, thereby signaling enhanced transcription of phase 2 genes (*5*, *6*); and (iii) Keap1, the sensor for inducers, a Kelch family multidomain repressor protein that is normally localized in the cytoplasm where it is tethered to the actin cytoskeleton (*7*−*10*). Keap1 binds Nrf2 very tightly and promotes its proteasomal degradation (*11*, *12*) by functioning as an adaptor for Cul3-based E3 ligase (*13*−*15*). Inducers disrupt the Keap1−Nrf2 complex, thereby releasing Nrf2 for translocation to the nucleus and activation of the transcription of phase 2 genes.

On the basis of its primary structure, Keap1 has five distinct domains: (i) NTR, N-terminal region, amino acids 1−60; (ii) BTB (broad complex, Tramtrack, Bric-á-brac), amino acids 61−179; (iii) IVR, intervening region, amino acids 180−314; (iv) DGR, double glycine repeat, also known as Kelch domain, amino acids 315−598; and (v) CTR, C-terminal region, amino acids 599−624. The BTB domain is an evolutionary conserved protein−protein interaction

[†] This work was suported by generous gifts from the Lewis B. and Dorothy Cullman Foundation, the McMullan Family Fund, the Barbara Lubin Goldsmith Foundation, grants from the National Cancer Institute, Department of Health and Human Services (Grant CA 94076), and from the American Institute for Cancer Research.

* To whom correspondence should be addressed: Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, 725 N. Wolfe Street, WBSB 406, Baltimore, MD 21205. Telephone: 410-955-3503. Fax: 410-502-6818. E-mail: adkostov@jhmi.edu.

[1] Abbreviations: ARE, antioxidant response element; CTR, C-terminal region of Keap1; Dex-mes, dexamethasone 21-mesylate; DGR, double glycine (Kelch) region of Keap1; DPDS, 4,4′-dipyridyl disulfide; IVR, intervening region of Keap1; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor-erythroid 2-related factor 2; NTR, N-terminal region of Keap1; PAR, 4-(2-pyridylazo)resorcinol; SF, sulforaphane; TCEP, Tris(2-carboxyethyl)phosphine.

10.1021/bi047434h CCC: $30.25
Published on Web 04/16/2005

motif that often promotes dimerization. Indeed, dimerization through the BTB domain is required for the ability of Keap1 to sequester Nrf2 and prevent transcription of phase 2 genes (*16*). The IVR is not only a particularly cysteine-rich region (8 cysteine residues among 134 amino acids) but is also the domain that contains the most reactive cysteine residues of Keap1 and appears to function as a regulatory domain for the function of the entire protein. The Kelch domain of Keap1 binds both F-actin and Nrf2 (*7*). Recently, the crystal structure of the Kelch domain of human Keap1 has been solved (*17*). It forms a classical 6-bladed β propeller with multiple potential protein-binding sites. Interestingly, the N and C terminus of the protein binding Kelch domain together form one of the blades of the propeller implying, in the context of the full-length protein, that the preceding IVR and the subsequent CTR of Keap1 must be in close proximity.

Overexpressed and purified murine Keap1 reacts directly with inducers in isolated systems, and the avidity of these reactions parallels the inducer potencies (*18*). Closer examination of the reactivity of Keap1 established that four (C^{257}, C^{273}, C^{288}, and C^{297}) of the 25 cysteine residues of murine Keap1 are particularly reactive with dexamethasone mesylate (an inducer and irreversible reagent for thiol groups). This finding suggested that these residues of the IVR of Keap1 were the ultimate targets for chemical reaction with inducers. Single and multiple mutations of these and other cysteine residues of Keap1 established that modification of either C^{273} or C^{288} but not of any other cysteine residues abrogated the capacity of Keap1 to repress the activity of Nrf2 (*19*−*21*). It appears that any type of modification of C^{273} and C^{288} (e.g., alkylation with inducers, participation in disulfide linkages (*19*), and possibly higher order oxidation states or even amino acid substitution) leads to the inability of Keap1 to bind, repress, and facilitate the degradation of Nrf2.

In an attempt to resolve the enigma of what makes C^{273} and C^{288} (among a total of 25 cysteine residues in murine Keap1) critical for the function of this protein, we considered the possibility that these residues might be involved in metal coordination. In recent years, there has been increasing recognition of the role of cysteine in protein structure and function, including proteins involved in signal transduction and those that sense oxidative stress (*22*−*24*). Unmodified and modified cysteine residues with sulfur in various oxidation states have been identified in many proteins, including thiol, disulfide, sulfenic, sulfinic, and sulfonic acids, disulfide-*S*-oxides, and sulfur-centered radicals (*25*, *26*). In addition to its redox reactivity, cysteine can serve as a ligand for metal coordination, and this unique property can also be finely tuned by changes in the oxidation state of its sulfur. Furthermore, when modulation of sulfur redox activity and metal binding occur within the same protein, redox control of metal binding and metal control of sulfur redox activity become possible (*26*).

In this paper, we establish that recombinant murine Keap1 is a zinc metalloprotein and determine how the kinetics and stoichiometry of zinc binding govern the susceptibility of these thiol groups to the reaction with inducers. Our experiments show that Keap1 is an example of a protein whose functions are regulated by ligand binding that depends on the reactivity of its cysteine thiols.

EXPERIMENTAL PROCEDURES

Protein Overexpression and Purification. Escherichia coli BL21(DE3) was transformed with a pET vector bearing a sequence that encoded either wild-type or mutant murine Keap1 (*18*). Mutant constructs were prepared by PCR and standard recombination techniques as described before (*19*), and the authenticity of each construct was confirmed by sequencing. To overexpress each protein, *E. coli* was grown in a mineral salts medium plus glucose essentially as described previously (*18*). The final cell pellets were stored at −70 °C until use. For certain experiments, the growth medium was supplemented with either 10−40 μM $ZnCl_2$ or 5−15 μM $CoCl_2$.

Crude extracts were prepared by sonication as described previously, except: (a) the buffer used was 50 mM Tris-HCl and 5.0 mM dithiothreitol (DTT) at pH 8.0 (no EDTA), although for a few extracts the DTT was replaced with either 2.5 mM Tris(2-carboxyethyl)phosphine (TCEP) or 20 mM 2-mercaptoethanol; and (b) the buffer was gassed with argon before use, and sonication was done under an atmosphere of argon. Initial purification of Keap1 was by precipitation of the protein after removal of the reducing agent and freezing at −70 °C. The precipitated protein was redissolved in 25 mM Tris-HCl at pH 8.0 containing the same reducing agent as used in the extract and under an atmosphere of argon. The once-precipitated protein was further purified by gel filtration on a 10 × 300 mm Superdex 200 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, and 0.01% Tween 20 at pH 8.0, at a flow rate of 0.5 mL/min, and the protein peak corresponding to the Keap1 dimer was collected and concentrated in a Centricon Plus-20 concentrator (Amicon-Millipore). The concentrated protein was stored at −70 °C under an atmosphere of argon. For some purposes, the protein concentration was determined by the Bradford assay (*27*) with crystalline BSA as a standard. For spectroscopic studies involving calculations of stoichiometry, the protein concentration was determined using a molar absorption coefficient of 80 080 M^{-1} cm^{-1} at 280 nm that was determined from its tyrosine, tryptophan, and cysteine (27, 8, and 25 residues per subunit, respectively) content and its absorbance change in guanidinium-HCl (*28*). Although the value obtained by the Bradford method was 13.8% higher, it provided a convenient assay that was corrected by dividing the protein concentration by 1.138.

Cross-Linking of Keap1. Cross-linking was performed in 25 mM HEPES, 100 mM NaCl, 0.01% Tween 20, and 2.5 mM TCEP at pH 8.0 with 0.25 μM Keap1 and 0.05% glutaraldehyde in 200-μL reaction volumes in an atmosphere of argon. At the end of the incubation, the reaction was terminated with 0.5 M Tris-HCl at pH 8.0 that also contained 0.3% SDS. Samples were then analyzed by SDS−PAGE (*29*).

[^{3}H]Dexamethasone 21-Mesylate (Dex-mes) Labeling. Aliquots of Keap1 were incubated with 5 mM DTT for 60 min at 0 °C, and then each aliquot was passed sequentially through 2 NAP-10 columns equilibrated with 20 mM Tris-HCl and 0.005% Tween 20 at pH 8.0, which had been gassed with argon. The final volume of the eluted protein was 1.5 mL and contained approximately 50 pmol of Keap1 in 100 μL. Aliquots of 100 μL of this Keap1 were incubated with

10 μL of chelating agents or H_2O for 30 min at 25 °C, and then 10 μL of [^{3}H]Dex-mes (25 pmol, about 600 000 CPM) was added to each reaction and incubation was continued for the indicated times. Dex-mes binding was terminated by the addition of 5 μL of 100 mM DTT; each reaction mixture was placed on an NAP-10 column and eluted, and the eluates were counted, as described previously (*18*).

Metal Analysis. Aliquots of the concentrated Keap1 dimer were each passed successively through two NAP-10 gel-filtration columns equilibrated with 5 mM Tris-HCl at pH 8.0 prepared in plasma-grade water (Omni*Trace* Ultra, EM Science). The final volume of the protein solutions was 1.5 mL. DTT was added to a final concentration of 1.0 mM, and the protein concentration was determined by Bradford assay and was usually between 0.1 and 0.3 mg/mL. An aliquot of the Superdex 200 column buffer was treated in an identical manner and served as a buffer blank. Metal content was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) at the Chemical Analysis Laboratory, University of Georgia, Athens, GA.

Preparation of Zinc-Reconstituted Keap1. A total of 2 μL of 2 mM $ZnCl_2$ was mixed with 2 μL of 2 mM DTT to form the Zn−DTT chelate complexes. To this, a stoichiometric amount of Keap1 (20 μM) was added under argon, and the mixture was incubated for 30 min at 25 °C in a final volume of 200 μL in metal-free, argon-saturated buffer (10 mM Tris-HCl at pH 8.0 and 0.005% Tween 20). Unbound zinc and DTT were then removed on an NAP-10 gel-filtration column that had been equilibrated with the same buffer, and the protein was eluted into an anaerobic quartz vial under argon.

Spectroscopic Determination of the Zinc Content of Keap1 and the Association Constant for Zinc Binding. Zinc was determined spectrophotometrically from the absorption change at 500 nm of the zinc complex with the chelator 4-(2-pyridylazo)resorcinol (PAR) in metal-free 10 mM Tris-HCl buffer at pH 8.0, containing 0.005% Tween 20. The concentration of the resulting PAR_2−Zn complex was calculated using $a_m = 66\,000\ M^{-1}\ cm^{-1}$ (*30*). For determination of the total zinc content in Keap1, PAR was first added (final concentration of 100 μM) to Keap1, followed by the sulfhydryl reagent 4,4′-dipyridyl disulfide (DPDS) (final concentration of 100 μM). This led to complete release of zinc from the protein and its capture by PAR. To determine the number of cysteines involved in metal binding, successive 2-μL aliquots of DPDS were added to the Keap1 solution in the presence of PAR and the release of zinc was monitored by the increase in absorbance at 500 nm as described by Jakob et al. (*31*).

For determination of the zinc-binding constants (K_a), 4.5 μM Keap1 was permitted to react with different concentrations of PAR (25−200 μM). After equilibrium was reached, the amount of zinc bound to PAR was calculated. DPDS (100 μM) was then added to release the remaining zinc bound to Keap1. The K_a was calculated by the equations shown in the Results, using the published second-order association constant K_a (PAR) $= 2 \times 10^{12}\ M^{-2}$ (*30*).

Cobalt Incorporation into Keap1. Metal-free Keap1 was obtained by the purification procedure described in Dinkova-Kostova et al. (*18*) in which 5 mM EDTA was included in the buffers that were used for cell lysis, precipitation of the crude extract, and solubilization of the pellet. The resulting protein had less than 0.05 mol of zinc/mol of protein as determined by the PAR assay. Subsequent HPLC purification of the Keap1 dimer (see above) was carried out in the absence of EDTA. Cobalt was titrated from a fresh 0.5 mM stock solution of $CoCl_2$ in 2-μL aliquots into metal-free Keap1 (35.5 μM) in 10 mM Tris-HCl at pH 8.0 in a final volume of 200 μL. The absorption spectra were obtained immediately after addition of each aliquot of $CoCl_2$ using a double-beam spectrophotometer. When full saturation with $CoCl_2$ was reached as judged by the approach to an absorption plateau at 640 nm, zinc was added in 2-μL aliquots of 1 mM $ZnCl_2$, up to a final concentration of 40 μM, and the absorptions were monitored.

Fluorescence of Keap1. Fluorescence measurements were carried out on a Perkin−Elmer LS50 spectrofluorimeter. The intrinsic tryptophan fluorescence of 0.8 μM Keap1 was first obtained in 2 mL of 20 mM Tris-HCl at pH 8.0 using an excitation wavelength of 295 nm. The excitation and emission slit widths were set at 8 nm. Measurements of the fluorescence quenching of Keap1 were done after the addition of 2 μL of 3 mM DPDS (final concentration of 3 μM). Fluorescence measurements under identical conditions were also obtained for denatured Keap1 (with 6 M urea or 4 M guanidinium-HCl buffered with Tris) as well as a mixture of all of the amino acids comprising Keap1 in their equivalent molar ratios.

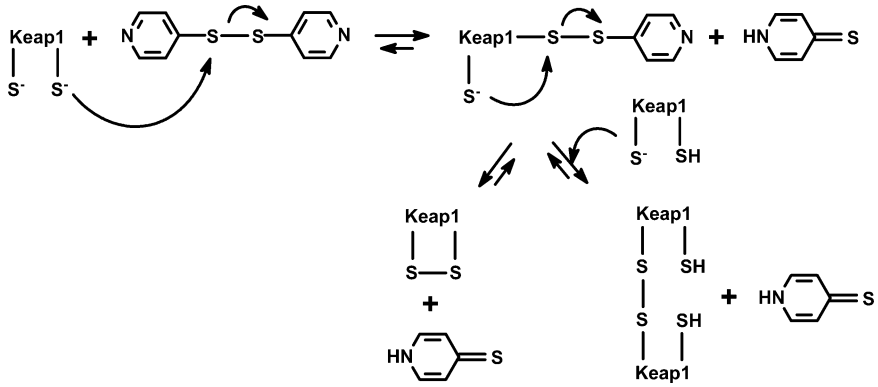
Fluorescence of 4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic Acid (Bis-ANS). Bis-ANS (Molecular Probes, Eugene, OR) was used to probe the hydrophobic surfaces of Keap1. Keap1 (1 μM) was added to a solution of bis-ANS (3.75 μM) in 20 mM Tris-HCl at pH 8.0 in 2.0 mL, and the fluorescence emission spectrum was monitored from 400 to 600 nm using an excitation wavelength of 395 nm and excitation and emission slit widths of 5 nm.

Temperature. All experiments were done at ambient temperature (23−25 °C).

RESULTS

Purified Recombinant Keap1 is a Dimeric Protein. Our previous unexpected finding that addition of 1 equiv of DPDS to Keap1 gave rise to 2 equiv of pyridinethione (*18*) suggested that, as soon as the first Keap1-pyridyl mixed disulfide was formed, this bond was attacked by another cysteine thiolate ion on the protein to form either an intra- or intermolecular protein disulfide (Scheme 1). Subsequent experiments established the formation of disulfide-linked dimers of Keap1 when cells, transiently transfected with a construct encoding for Keap1, were treated with various inducers (*19*). To determine the oligomerization state of Keap1 in our recombinant protein preparations, murine Keap1 was overexpressed and purified as described previously by freezing sonic supernatant fractions from *E. coli* in the presence of salt and redissolving the resulting precipitate in buffers containing DTT (*18*). The protein was further purified by gel-filtration chromatography on Superdex 200 sizing column under reducing conditions, and the peak migrating as a dimer (MW ∼ 150 000) was collected and subjected to cross-linking with glutaraldehyde. After 3, 10, or 30 min, the reaction was stopped and the protein was denatured and analyzed by SDS−PAGE. As shown in Figure 1, the appearance of a band corresponding to a chemically

Scheme 1: Thiol-disulfide Interchange Reaction of Keap1 with DPDS[a]



[a] A nucleophilic attack on the electrophilic disulfide of DPDS by a cysteine thiolate ion of Keap1 leads initially to the formation of a Keap1-pyridyl mixed disulfide that in turn is rapidly attacked by another cysteine thiolate of the protein to form either an intra- or intermolecular protein disulfide.

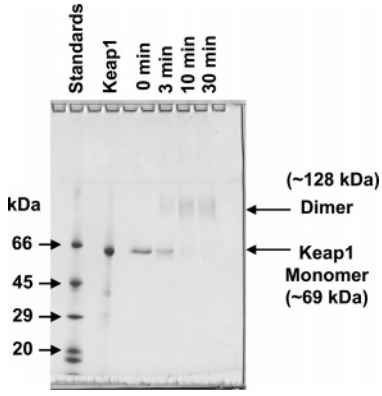


FIGURE 1: SDS−PAGE analysis of Keap1 after cross-linking with glutaraldehyde. Cross-linking of Keap1 (0.25 μM) with 0.05% glutaraldehyde was carried out in 25 mM HEPES, 100 mM NaCl, 0.01% Tween 20, and 2.5 mM TCEP at pH 8.0. The samples were analyzed by SDS−PAGE after 3, 10, or 30 min of incubation. Note the progressive dimer formation.

cross-linked dimer of Keap1 was detected 3 min after addition of glutaraldehyde. Moreover, after 30 min, only the dimeric species was present in the sample and no monomer could be detected. This experiment further confirmed that under these conditions Keap1 exists as a dimer even at the lowest concentration tested (0.25 μM). *All subsequent experiments reported below were performed with the dimeric form of Keap1 obtained by chromatography on a sizing column.*

Metal Analysis of Dimeric Keap1. Because Keap1 is the sensor for electrophiles and oxidants, it is important to understand why some of its 25 cysteine residues are much more reactive than others (*18*, *19*). In the absence of a complete crystallographic structure, we have speculated that particularly reactive cysteines are flanked by basic amino acids, which decrease their pK_a values (*32*). Another possibility is that such reactive cysteines coordinate a metal that keeps them in their reactive thiolate state. Both conditions could contribute to selective reactivity. To address the second possibility, the metal content of the Keap1 dimer purified in the presence of TCEP was analyzed by ICP-OES. The only metal that was consistently present in such preparations was Zn. Analysis of three different preparations revealed that those obtained from *E. coli* grown in minimal mineral medium prepared in water that was not deliberately deionized and contained less than 1 μM Zn (as determined by ICP-OES) only had a substoichiometric amount of zinc (0.23 ± 0.07 mol of Zn^{2+}/subunit mol of Keap1). This has been observed for other overexpressed proteins (*31*). In contrast, cells grown in medium supplemented with zinc (i.e., in the presence of 10−40 μM $ZnCl_2$) express Keap1 that has a nearly stoichiometric amount of zinc (0.91 ± 0.34 mol of Zn^{2+}/subunit mol of Keap1).

[^{3}H]Dex-mes Labeling. In a previous publication we demonstrated that tritiated Dex-mes, an electrophile and inducer of phase 2 enzymes, labeled Keap1 primarily at only 5 of the 25 cysteine residues: four IVR (intervening region) cysteines C^{257}, C^{273}, C^{288}, and C^{297} and the carboxy terminal cysteine C^{613} (*18*). Consequently, labeling experiments were carried out using [^{3}H]Dex-mes to determine if we could obtain insight into which cysteine residues might be involved in metal binding. When purified Keap1 was labeled in the presence and absence of various chelating agents, binding of the steroid was stimulated about 2-fold by EDTA, EGTA, and TPEN, whereas 1,10-phenanthroline and the Fe^{3+} chelator deferoxamine had little or no effect (Figure 2A). These results suggest that one or more of the five Dex-mes-labeled cysteines might be involved in metal binding and that the presence of a chelator might facilitate metal displacement in response to inducers. To further explore this possibility, the binding of [^{3}H]Dex-mes to the wild-type Keap1 in the presence or absence of EDTA was compared to that of a mutant Keap1 in which the four critical IVR cysteines had been replaced by alanine. In the absence of EDTA, binding to the mutant was reduced to only about 22% of that of the wild type at each time point, confirming our previous observations (*18*, *19*) of the high reactivity of these residues with Dex-mes (Figure 2B). In the presence of EDTA, the mutant showed a similar percentage increase of steroid binding; however, the absolute amount of binding was still far less than the wild type. A similar comparison was done between the wild-type Keap1 and a mutant in which the three carboxy terminal cysteines (C^{613}, C^{622}, and C^{624}) had been replaced by alanine. In this case, the mutant bound Dex-mes as well as the wild type in the absence of EDTA, and binding in the presence of EDTA was stimulated equally well if not better (Figure 2B). Although these binding experiments confirmed the reactivity of the C^{257}, C^{273}, C^{288}, and C^{297} IVR cysteines with Dex-mes, they did not provide

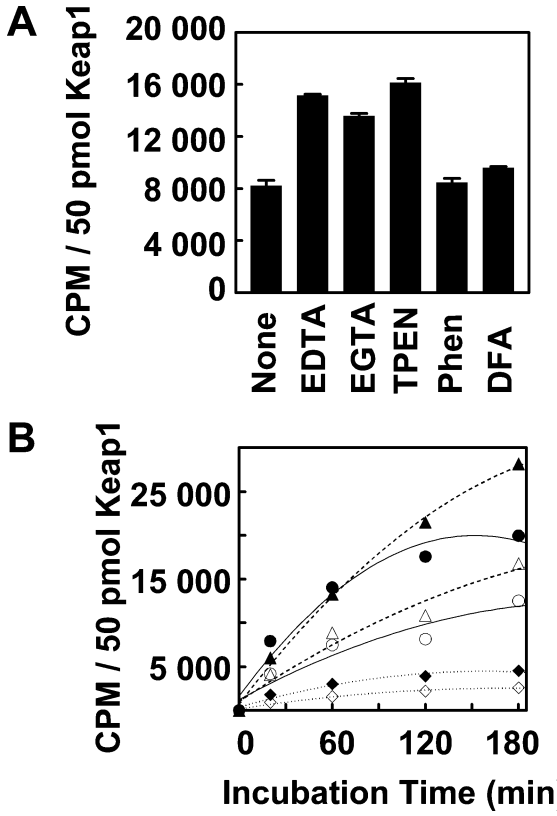


FIGURE 2: [^{3}H]Dex-mes binding to Keap1 is affected by metal chelating agents. (A) Keap1 (50 pmol) was preincubated for 30 min in 100 μL of 20 mM Tris-HCl, and 0.005% Tween 20 at pH 8.0 with various metal chelating agents at a final concentration of 41 μM. Then, [^{3}H]Dex-mes (25 pmol) was added, and incubation continued for 60 min. Unbound [^{3}H]Dex-mes was removed by gel filtration, and the radioactivity in the protein fraction was determined. EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine; Phen, 1,10-phenanthroline; DFA, deferoxamine. (B) Time course of incubation of [^{3}H]Dex-mes (25 pmol) with wild-type Keap1 (● and ○), C257A, C273A, C288A, C297A quadruple mutant (◆ and ◇), or C613A, C622A, C624A triple mutant (▲ and △) in the presence (●, ◆, and ▲), or absence (○, ◇, and △) of 100 μM EDTA. For each type of Keap1, the initial reaction rate was higher in the presence of EDTA. Whereas mutations in the CTR (C^{613}, C^{622}, and C^{624}) had only small effects on Dex-mes binding, the reaction was much slower with the IVR mutant (C^{257}, C^{273}, C^{288}, and C^{297}).

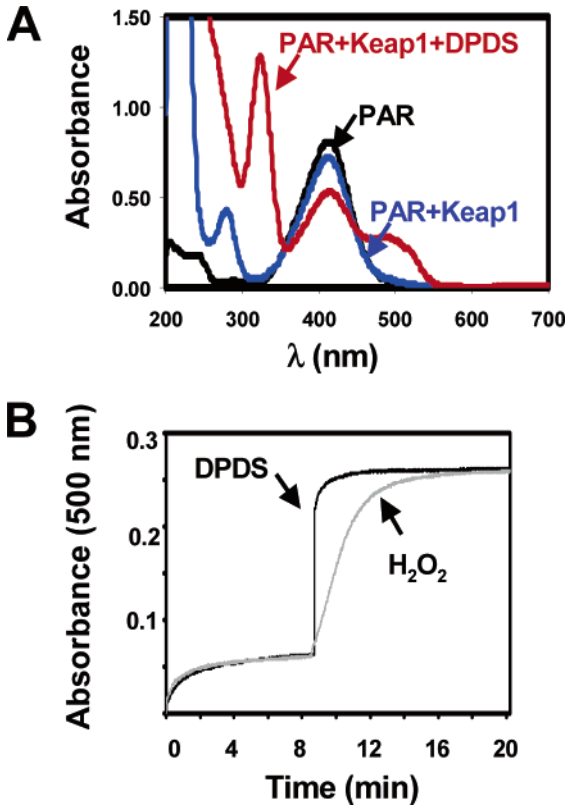


FIGURE 3: Cysteine residues of Keap1 serve as ligands for zinc coordination. (A) UV−vis spectrum of PAR (25 μM) in the absence (black trace) or presence of 5 μM zinc-saturated reduced Keap1 (blue trace) or Keap1 that has been reacted with 100 μM DPDS (red trace). The increase in absorbance at 500 nm corresponds to the formation of the PAR_2−Zn complex, while the increase in absorbance at 325 nm corresponds to the formation of the pyridinethione. (B) Keap1 (5 μM) was incubated with 100 μM PAR in 20 mM Tris-HCl at pH 8.0. The increase in absorbance at 500 nm of the PAR_2−Zn complex was measured with time. After a plateau was reached, inducers (100 μM 4,4′-dipyridyl disulfide or 500 μM H_2O_2) were added to release the remaining zinc, which then became available for complex formation with PAR. The final plateau absorbance of 0.26 corresponds to 4 μM PAR_2−Zn.

unequivocal evidence that these residues are involved in metal binding.

Experimental Strategy for the Quantification of Zinc Binding and Thiol Groups Participating in Zinc Binding. To obtain quantitative information on the stoichiometry of zinc binding to Keap1 and the participating cysteine thiols, two reagents were used alone or in combination: (i) the metal chelating azo dye PAR, which binds zinc to form a complex (PAR_2−Zn) that absorbs light strongly at 500 nm with a_m = 66 000 M^{-1} cm^{-1} (*30*), and (ii) DPDS, which upon reaction with thiol groups gives rise to a pyridinethione (*33*) that absorbs at 325 nm with a_m = 20 000 M^{-1} cm^{-1} (Scheme 1 and Figure 3A).

Incubation of Keap1 (2.5−10 μM) with 100 μM PAR released ~25% of the total zinc as monitored by the increase in absorbance at 500 nm. The remaining zinc could be released only after the addition of an excess of inducers. Three different inducers were added: sulforaphane (100 μM), H_2O_2 (500 μM), and DPDS (100 μM). The results were essentially the same with all three inducers, and the total amount of zinc released was proportional to the protein concentration (Figure 3B). In the presence of excess inducer, the total amount of zinc released into solution and chelated by PAR was the same as the total amount in the protein preparation as determined by ICP-OES analysis. Because the only common property among these inducers is their ability to react with sulfhydryl groups, this result indicated that at least the major fraction of the metal is coordinated by cysteine residues.

Keap1 and PAR Compete for Zinc Binding. The previous experiment demonstrated that the zinc that is released from Keap1 upon inducer treatment can be chelated by PAR. The next question was whether Keap1 can compete with PAR for zinc binding. For this purpose, 5 μM zinc-deficient Keap1 (0.2 mol of Zn^{2+}/subunit mol of Keap1) was incubated with 100 μM PAR (Figure 4). Upon addition of 5 μM $ZnCl_2$, the resulting increase in absorbance at 500 nm accounted for the formation of only 1.4 μM of the PAR_2−Zn complex, indicating that only 23% of the added zinc was available to the chelator and the majority of added zinc became bound to Keap1. To verify this assumption, treatment with an excess

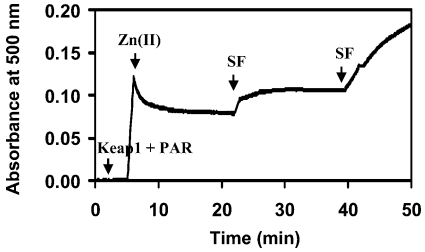


FIGURE 4: Keap1 competes with PAR for zinc binding. A total of 5 μM zinc-deficient Keap1 was incubated with 100 μM PAR in 20 mM Tris-HCl at pH 8.0. Upon addition of 5 μM $ZnCl_2$, the increase in absorbance at 500 nm corresponded to only 1.4 μM PAR_2−Zn complex. Two successive additions of sulforaphane (100 μM) at 22 and 40 min released the zinc from Keap1, resulting in an increase of absorption at 500 nm and, after completion of the reaction (not shown), to the final formation of 5.9 μM PAR_2−Zn complex.

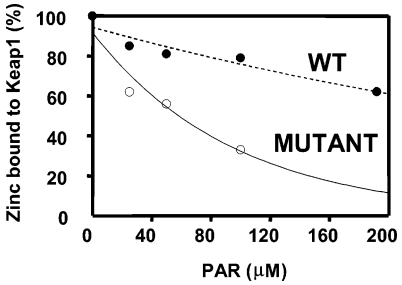


FIGURE 5: Binding affinity of Keap1 to zinc. The zinc-binding constants of the wild type (●, - - -) and C257A, C273A, C288A, C297A quadruple Keap1 mutant (○, —) were determined in competition with PAR. Keap1 (4.5 μM) was incubated without or with increasing concentrations of PAR, and after reaching equilibrium, the amount of PAR_2−Zn complex was calculated. DPDS was then added to determine the amount of zinc that was still bound to Keap1.

of sulforaphane followed by denaturation with 0.1% SDS (to decrease the resulting slight turbidity of the solution and to allow completion of the measurements) resulted in complete release of zinc (4.5 μM) from Keap1 and its capture by PAR giving rise to a total of 5.9 μM of the PAR_2−Zn complex, in reasonable agreement with the total amount of zinc present in the system.

Binding Affinity of Keap1 for Zinc. We employed the PAR assay in conjunction with DPDS, an inducer and a sulfhydryl reagent, to explore the zinc-binding properties of Keap1. For this purpose, we needed a homogeneous preparation of metal-saturated protein. Initial attempts to add even stoichiometric amounts of zinc (in the form of $ZnCl_2$) directly to purified Keap1 dimer under reducing conditions (DTT) at pH values from 7.2 to 8.2 and at different temperatures (from 4 to 37 °C) failed, as the solutions became turbid, and ultimately the protein precipitated even when the buffer was saturated with argon. However, precipitation did not occur when Keap1 was added to the preformed PAR_2−Zn complexes or when zinc was added to a solution containing both Keap1 and the chelator PAR. This observation suggested that one approach to introducing zinc into Keap1 successfully could be in the form of zinc chelate complexes. Indeed, zinc reconstitution of Keap1 was accomplished by first mixing $ZnCl_2$ and DTT in an equimolar ratio (to form Zn−DTT chelate complexes) and then adding stoichiometric amounts of protein to this mixture in an anaerobic atmosphere (under argon). Excess DTT and unbound metal were then removed on an NAP-10 gel-filtration column that was equilibrated with metal-free, argon-saturated buffer, and the protein was eluted into an anaerobic quartz vial under argon. The metal content of this zinc-reconstituted preparation was then determined by the PAR spectrophotometric assay. We found that Keap1 had bound a stoichiometric amount of zinc at all time points of incubation (30, 60, or 90 min).

The zinc-binding constant of Keap1 was determined in competition with PAR as described by Zhou et al. (*34*). The reducing agent was removed from Keap1 under anaerobic conditions (in an argon atmosphere) to prevent cysteine oxidation. Keap1 (4.5 μM) was then incubated with increasing concentrations of PAR (25−200 μM) at pH 7.2. The absorbance at 500 nm was measured, and from it, the amount of [PAR_2−Zn] corresponding to the amount of zinc extracted from Keap1 by PAR was calculated. As can be seen in Figure 5, PAR was able to extract zinc from Keap1 in a concentration-dependent manner. It should be noted that, while the release of zinc from the wild-type Keap1 was linearly dependent on the concentration of the competitor, the same relationship was exponential for the zinc release from the mutants. When the absorbance at 500 nm became constant with time, an excess of DPDS (100 μM) was added to release completely the remaining zinc that was still bound to Keap1. The resulting total increase in absorbance corresponded to the concentration of the total zinc present in the system. The association constants (Table 1) of Keap1 wild type and its mutants for zinc (K_a) were determined according to the following equations:

Table 1: Binding Constants of Wild Type and Mutants of Keap1 for Zinc[a]

Keap1 species	K_a (M^{-1})
wild type	1.02 (±0.19)[b] × 10^{11}
C273A	1.24 (±0.31) × 10^{10}
C288A	1.03 (±0.33) × 10^{10}
C273A, C288A	5.55 (±2.55) × 10^{9}
C257A, C273A, C288A, C297A	2.70 (±1.27) × 10^{9}

[a] Keap1 wild type or mutants (4.5 μM) were permitted to react with a series of different concentrations of PAR (25−200 μM) in metal-free 10 mM Tris-HCl buffer at pH 8.0 containing 0.005% Tween 20. After equilibrium was reached, the amount of zinc bound to PAR was calculated from the absorbance at 500 nm corresponding to the PAR_2−Zn complex using $a_m = 66\,000\ M^{-1}\ cm^{-1}$ (*30*). DPDS (100 μM) was then added to determine the amount of zinc that remained bound to Keap1. The K_a was calculated by the equations shown under the Results, using the published association constant K_a (PAR) = 2 × 10^{12} M^{-2} (*30*). [b] ±SD based on 3−4 different concentrations of PAR.

$$K_{a(PAR)} = \frac{[PAR_2{-}Zn]}{[Zn_{free}][PAR_{free}]^2} = 2 \times 10^{12}\ M^{-2}$$

$$K_{a(Keap1)} = \frac{[Keap1{-}Zn]}{[Zn_{free}][Keap1_{free}]}$$

$$K_{a(Keap1)} = \frac{[Keap1{-}Zn]K_{a(PAR)}[PAR_{free}]^2}{[PAR_2{-}Zn][Kepa1_{free}]}$$

The association constant (K_a) of wild-type Keap1 for zinc was calculated to be 1.02 (±0.19) × 10^{11} M^{-1}. It is about 1 order of magnitude higher than the zinc binding constant for the neural zinc finger factor 1 [K_a = 1.4 × 10^{10} M^{-1}]

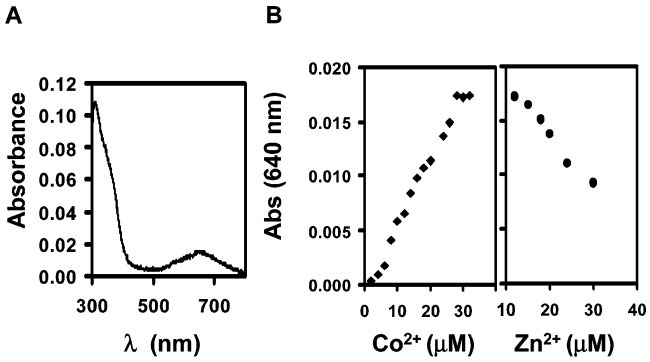


FIGURE 6: Keap1 can accommodate cobalt into its metal-binding site. (A) Absorption spectrum of reduced Keap1 (35.5 μM) treated with 40 μM $CoCl_2$ at pH 8.0. The spectra of reduced metal-free Keap1 and $CoCl_2$ have been subtracted. (B) Absorption increases at 640 nm (left panel) upon addition of 2-μL aliquots of 0.5 mM $CoCl_2$ to 35.5 μM solution of Keap1 in a 200-μL volume and absorption decreases (right panel) upon addition of 2-μL aliquots of 1 mM $ZnCl_2$.

(*35*). In contrast, all mutant proteins bound zinc with lower affinity (Table 1). Thus, the single cysteine (C273A or C288A) mutants have an order of magnitude lower affinities to zinc, and the binding affinity of the doule cysteine mutant (C273A and C288A) is about 20 times lower than that of the wild type. Even weaker (by nearly 100-fold) is the affinity of the quadruple mutant (C257A, C273A, C288A, and C297A) implying that some of the reactive cysteine residues from the IVR are involved in metal coordination possibly together with a fourth (hitherto unidentified) amino acid.

Keap1 Can Accommodate Cobalt in Its Metal-Binding Site. It is well-known that cobalt can replace zinc in the metal-binding sites of metalloproteins. Moreover, because zinc is spectroscopically silent, while cobalt complexes have distinctive optical absorption spectra, cobalt has been used to probe the zinc-binding sites of metalloproteins (*36*). When $CoCl_2$ was titrated into a solution containing reduced metal-free Keap1, a characteristic concentration-dependent change in the UV−visible spectrum of Keap1 was observed. Of note, unlike the addition of $ZnCl_2$ that caused precipitation of the protein, the addition of $CoCl_2$ under similar conditions did not. As can be seen in Figure 6A, in the spectrum of the cobalt-saturated protein, there is an absorbance maximum at 640 nm with $a_m = 420\ M^{-1}\ cm^{-1}$. There is also an absorption maximum at 310 nm and a shoulder at 360 nm. These spectral characteristics are similar to those of a cobalt-saturated peptide representing one of the zinc finger domains of the *Xenopus* transcription factor IIIA (*37*) and to the cobalt-saturated zinc finger domain of the metal responsive element-binding transcription factor 1 (*38*), both being zinc-binding proteins in which the metal coordination is tetrahedral and is provided by 2 cysteine and 2 histidine ligands (also known as C_2H_2 type of zinc finger proteins). Titration of small amounts of $CoCl_2$ into Keap1 led to a proportional increase in the absorbance at 640 nm until stoichiometric amounts of $CoCl_2$ were added (Figure 6B). Adding a 2-fold excess of cobalt did not lead to an additional change in absorbance. Titration of $ZnCl_2$ to cobalt-saturated Keap1 led to a decrease in the absorbance at 640 nm (Figure 6B), indicating that zinc can displace cobalt from Keap1. Because the amount of cobalt was in excess of the protein and therefore higher than the amount of zinc present in the system, this indicated that the affinity of Keap1 for zinc is higher than its affinity for cobalt. This conclusion was also supported by an experiment in which the growth medium of *E. coli* was supplemented with two different concentrations of $CoCl_2$ (5 or 15 μM) during the overexpression of Keap1. Metal analysis revealed that the resultant protein contained both zinc and cobalt (Table 2). In the absence of any metal supplementation, the purified Keap1 dimer contained 0.333 mol of Zn/mol of monomer. When the medium was supplemented with 5 μM $CoCl_2$, the total metal content of Keap1 was nearly the same; however, the protein had incorporated zinc and cobalt in almost equal amounts. With higher cobalt supplementation (15 μM), the cobalt content was slightly increased. These experiments showed that (i) cobalt can substitute for zinc in the metal-binding site of isolated Keap1 *in vitro* as well as *in vivo* during protein synthesis, (ii) cobalt is incorporated into Keap1 depending on metal availability, and (iii) the affinity of Keap1 for zinc is higher than for cobalt.

Table 2: Zinc and Cobalt Content of Keap1 Overexpressed in *E. coli* Grown in Salt Medium with Metal Supplementation[a]

metal supplementation of *E. coli* medium	mol of Zn/mol of Keap1 monomer	mol of Co/mol of Keap1 monomer
no metal	0.333	0
10 μM $ZnCl_2$	0.932	0
5 μM $CoCl_2$	0.149	0.124
15 μM $CoCl_2$	0.174	0.228

[a] Keap1 was overexpressed in *E. coli* BL21(DE3) grown in a salt medium (1 g of NH_4Cl, 1 g of KH_2PO_4, 3 g of K_2HPO_4, 0.3 g of Na_2SO_4, 0.05 g of $MgCl_2$, and 0.005 g of $CaCl_2$ per liter) and glucose (0.3%) without or with metal supplementation. The recombinant dimeric protein was purified, and its metal content was determined by inductively coupled plasma-optical emission spectrometry.

Keap1 Changes Conformation upon Reaction with Inducers. We have proposed that, upon inducer sensing, Keap1 undergoes a conformational change that leads to dissociation of Nrf2, allowing the nuclear translocation of this transcription factor and ultimate activation of the expression of phase 2 cytoprotective genes (*19*). Because Keap1 contains 8 tryptophan residues, 6 of which are located in the Kelch domain where binding of Nrf2 takes place, we examined the conformation of Keap1 by monitoring its intrinsic tryptophan fluorescence. An equimolar amount of tryptophan alone or equimolar amounts of the mixture of all of the amino acids comprising Keap1 have fluorescence spectra identical to the spectrum of the denatured protein (with SDS, urea, or guanidinium) with an emission maximum at 350 nm (Figure 7). In contrast, the fluorescence intensity of the native protein is 4 times higher, and its emission maximum is shifted to 337 nm, indicating that some of the tryptophan residues are located in a hydrophobic environment that is not accessible to fluorescence quenching by the solvent, in agreement with the crystal structure of the Kelch domain of human Keap1 in which the six tryptophan residues are part of a hydrophobic core at the interface between two blades of the propeller (*17*). When an inducer (DPDS or sulforaphane) was added to Keap1, substantial (25%) quenching of the tryptophan fluorescence was observed with a blue shift of the emission maximum to 344 nm, suggesting that inducer sensing leads to major structural rearrangements in the protein and a decrease in hydrophobicity.

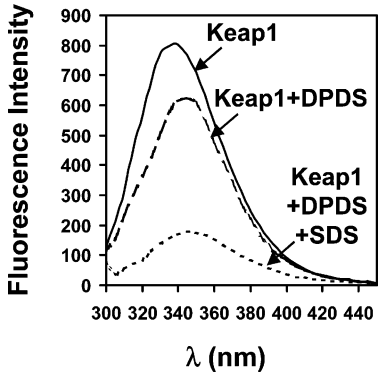


FIGURE 7: Intrinsic tryptophan fluorescence spectra of Keap1. Native Keap1 (0.8 μM) before (—) and after addition of DPDS (3 μM) (- - -) and after denaturation with SDS (0.3%) (•••). The emission spectra were recorded in 20 mM Tris-HCl at pH 8.0 using an excitation wavelength of 295 nm. The high tryptophan fluorescence of native Keap1 is quenched when the protein is treated with the thiol reagent DPDS and further when denatured with SDS. The fluorescence intensity of denatured Keap1 is identical to that of a solution of pure tryptophan of an equivalent concentration (8 tryptophan residues per subunit of Keap1).

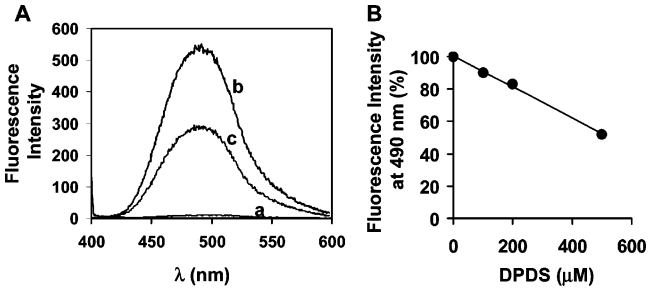


FIGURE 8: Disulfide bond formation causes a conformational change in Keap1, leading to a decrease in exposed hydrophobic surfaces. (A) Fluorescence spectra of 3.75 μM hydrophobic probe bis-ANS alone, trace a; bound to Keap1 (1 μM), trace b; and bound to Keap1 (1 μM) that had been reacted with DPDS (500 μM), trace c. (B) Linear relationship between the decrease in fluorescence intensity and the amount of DPDS added. Bis-ANS (3.75 μM) was incubated with Keap1 (1 μM), after which increasing concentrations of DPDS were added. The emission spectra were recorded in 20 mM Tris-HCl at pH 8.0 using an excitation wavelength of 395 nm. The bis-ANS probe becomes highly fluorescent when bound to Keap1, and this fluorescence is partially quenched after the reaction of Keap1 with DPDS.

We next used 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) to probe the hydrophobic surfaces of Keap1. Bis-ANS has a very low fluorescence in aqueous solvents with an emission maximum at ~520 nm, while when bound to hydrophobic surfaces of proteins, it exhibits a ~30 nm blue shift and a marked increase in fluorescence intensity (*39*, *40*). As can be seen in Figure 8A, the presence of Keap1 enhances by ~500-fold the fluorescence of free bis-ANS and shifts its emission maximum from 515 to 490 nm. Reaction with DPDS and hence disulfide formation and zinc release changes the binding of bis-ANS to Keap1 and dramatically decreases the fluorescence intensity of the probe in a concentration-dependent manner (Figure 8B). At 500 μM DPDS, the total decrease in fluorescence was ~50%, with no further change when higher concentrations of DPDS were added (not shown). This experiment confirmed that disulfide formation (and concomitant zinc release) leads to a conformational change in Keap1 with a decreased exposure of its hydrophobic surfaces.

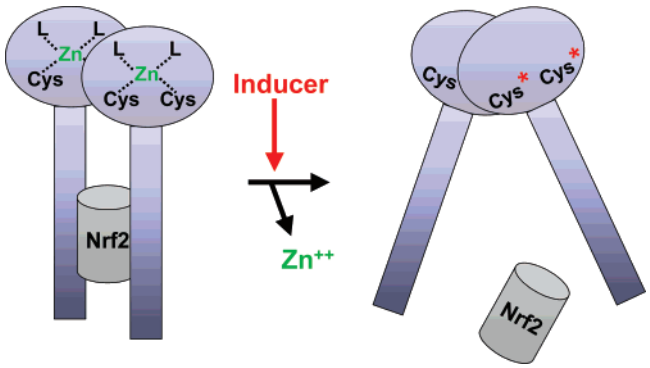
DISCUSSION

It is now widely believed that the major mechanism for upregulation of phase 2 proteins involves the Keap1—Nrf2—ARE signaling pathway and that stimulation of this pathway by a wide variety of inducers results in cellular protection against oxidant and electrophile stress. This work attempts to understand the critical initial event in this pathway: the interaction of inducers with the sensor that recognizes them and sets in motion the events leading to enhanced transcription of the cognate phase 2 genes.

We have suspected for many years that the intracellular sensor that recognizes the inducers contained very highly reactive cysteine thiol groups (*3*, *41*—*44*). Specific evidence for the participation of especially reactive thiols in the sensing of inducers was based on the chemistry of inducers: (a) The only recognizable common property of inducers, belonging to at least 10 different chemical classes, was their reactivity with thiol groups. (b) The potencies of inducers paralleled their reactivity with thiol groups. Thus, in inducers that are Michael reaction acceptors, the inducer potency was related to their reaction rate as electrophiles. (c) The very high inducer potency of trivalent arsenicals (e.g., phenyl arsenoxide) suggested the involvement of two vicinal thiol groups, possibly resulting in the formation of relatively stable cyclic thioarsenites. (d) The potency order of divalent cation inducers (Hg^{2+} > Cd^{2+} > Zn^{2+}) paralleled precisely their thiol reactivity. Although these observations suggested participation of critical thiol groups in the induction mechanism, the resultant chemical modifications of thiol groups comprised a variety of rather diverse reactions for different types of inducers, such as: (a) alkylation (Michael reaction acceptors; quinones), (b) oxidation (e.g., peroxides and hydroperoxides), and (c) direct reactivity with thiol/disulfide linkages (e.g., vicinal dithiols such as lipoic acid). This rather promiscuous signaling of induction provides plasticity for cellular responses to a variety of electrophile and oxidant stressors.

The identification of the intracellular sensor that recognizes and reacts with inducers became finally possible with the discovery of Keap1 by Yamamoto and colleagues (*7*). Murine Keap1 is a protein of 624 amino acids, including 25 cysteine residues, many of which are intrinsically highly reactive (to alkylation and oxidation and for metal binding) because they are flanked by basic residues that substantially lower their pK_a values (*32*). Preliminary experiments with recombinant proteins showed that inducers react much more avidly with Keap1 than Nrf2, making Keap1 a good candidate for the long sought inducer sensor. We subsequently demonstrated spectroscopically that Keap1 reacts directly with various inducers and identified five cysteine residues as most reactive. This goal was accomplished by chemical modification with Dex-mes, an inducer that reacts irreversibly with thiol groups (*18*). Interestingly, individual or collective mutations of two of these highly reactive cysteine residues, i.e., Cys^{273} and Cys^{288}, rendered Keap1 unable to repress Nrf2 (*19*—*21*). This finding led us to conclude that these cysteine residues are absolutely essential for the structural integrity of the repressor. One possibility to account for their selective reactivity is that they might be coordinating a metal. Involvement in metal coordination could keep the cysteine residues from being "hyper-reactive" under basal conditions

Scheme 2: Model for the Regulation of the Phase 2 Response[a]



[a] Under basal conditions, each monomer of dimeric Keap1 binds zinc that is coordinated in part by reactive cysteine residues (Cys), most likely Cys^{273} and Cys^{288} among them, and two other hitherto unidentified ligands (L). In this conformation, Keap1 binds Nrf2 and targets the transcription factor for degradation through the proteasome. Upon inducer sensing, the zinc is released and the reactive cysteine residues are modified by alkylation, oxidation, or thiol-disulfide interchange, thus leading to a conformational change that separates the Kelch domains and releases Nrf2 permitting its nuclear translocation and ultimately enhanced expression of phase 2 genes.

yet be "poised" to react with an inducer because they are already in their thiolate state and do not require deprotonation.

In this contribution, we report that recombinant murine Keap1 is a dimeric metalloprotein in which each monomer binds a stoichiometric equivalent of zinc with affinity comparable to the metal-binding affinity of known zinc finger proteins. As is the case with many other metalloproteins, Keap1 can also accommodate Co^{2+} in its metal-binding site both during protein synthesis in *E. coli* as well as *in vitro*, but its affinity for zinc is higher than that for cobalt. The spectral properties of the Co^{2+} complex suggest tetrahedral coordination. Reaction with inducers of various structural types leads to zinc release and cysteine modification causing a profound conformational change in Keap1 as evidenced by decreases in its intrinsic tryptophan fluorescence and exposed hydrophobic surfaces. Curiously, the interaction of Keap1 with inducers or Nrf2 occurs *in vitro* in the absence of metal, because all of the previous experiments were performed in the presence of EDTA (*18*). Furthermore, now we find that Keap1 is more reactive with Dex-mes *in vitro* in the presence of known zinc chelators, i.e., EDTA, EGTA, or TPEN but not the Fe^{3+}-chelator deferoxamine. Thus, it would appear that the metal plays a dual role: it protects its ligands from reacting under basal conditions yet keeps them poised for reaction upon inducer sensing.

A tentative mechanistic model (Scheme 2) for the consequences of the reaction of inducers with Keap1 suggests that these chemical modifications produced conformational changes in Keap1 that could result in weakening of the Keap1/Nrf2 binding interface. The present evidence for this proposal is (a) the complex of Nrf2 with Keap1 is disrupted by inducers; (b) the reaction of Keap1 with inducers is directly demonstrable spectroscopically; and (c) reaction of Keap1 with inducers results in modification of critical cysteine residues by alkylation (e.g., Dex-mes), oxidation (e.g., H_2O_2), or intermolecular disulfide-linked dimer formation (e.g., DPDS). In this paper, we demonstrate that the reaction of Keap1 with inducers depresses the tryptophan fluorescence of Keap1, an observation that is reasonably explained by a change in conformation resulting in exposure of these residues to a more hydrophilic environment. The new finding, described in this paper, that Keap1 is a Zn^{2+} metalloprotein (with a stoichiometry of approximately one metal atom per protein monomer) raises the issue of the possible functional role of zinc in the reaction of Keap1 with inducers and the relation of this process to the function of the critical thiol groups.

There are many zinc-thiol proteins, and one of the cooperative relations between cysteine residues and the metal ion is the resulting change in the nucleophilicity of the thiol groups of the cysteines, thereby modulating their oxidative lability and metal-binding affinities. This relation has been described by Giles et al. (*25*): "situations in which these three properties coincide in proteins, giving rise to redox control of cysteine catalytic activity, redox control of metal binding, and metal control of cysteine catalytic activity". The reciprocal relation between zinc and cysteine thiols in Keap1 receives experimental support from our finding that the reaction of Keap1 with inducers of very different types resulted in the invariable displacement of zinc from the protein. Most importantly, the findings described in this contribution suggest that the inducer-sensitive "zinc-cysteine partnership" provides a fine and elegant controlling mechanism that regulates the interaction of Keap1 and Nrf2 and ultimately the expression of phase 2 genes.

Changing the oxidation state of critical cysteines, the so-called "redox switch", represents a facile, subtle, and elegant way to modulate the activity of proteins that sense and cope with oxidative and disulfide stress (*23*, *24*). The *E. coli* heat-shock protein Hsp33 operates by a redox zinc switch, whereby oxidation of the four cysteine ligands of its zinc site to form two intramolecular disulfides leads to zinc release, domain unfolding, dimerization, and ultimately activation of its chaperone function (*24*, *45*). The antisigma factor RsrA from *Streptomyces coelicolor* also has a zinc thiolate redox switch: in its reduced state, RsrA binds zinc and has a conformation that allows binding and repression of the transcription factor sigma, whereas disulfide stress causes disulfide bond formation, zinc release, and ultimately RsrA to acquire a conformation that does not allow binding to the sigma factor (*23*, *46*, *47*). These and many other examples of zinc thiolate redox switches demonstrate that the redox-sensitive "zinc-cysteine partnership" is essential not only for the structure and function of some zinc-binding proteins but also for controlling their interactions with other proteins, DNA, and lipids, thus serving a critical role in the association and dissociation of macromolecular complexes (*48*).

In conclusion, the regulation of the cytoprotective phase 2 gene response involves the chemical modification by inducers of at least two (Cys^{273} and Cys^{288}) cysteine residues of the IVR of Keap1, which is the primary cellular sensor for these inducers. Keap1 contains thiol-bound zinc (approximately 1 mol per subunit), which is displaced by the reaction with inducers or other classical sulfhydryl reagents. These reactions with inducers result in profound conformational changes in Keap1, manifested by large decreases in the tryptophan fluorescence of the protein and the capacity to quench the fluorescence of a dye probe that becomes

intensely fluorescent when bound to hydrophobic residues of native Keap1.

ACKNOWLEDGMENT

We are very grateful to Paul Talalay for advice on experimental design and interpretation and in preparation of the manuscript, to Pamela Talalay for valuable editorial consultation, and to Albert S. Mildvan and Philip A. Cole for helpful discussions.

REFERENCES

1. Talalay, P., Dinkova-Kostova, A. T., and Holtzclaw, W. D. (2003) Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis, *Adv. Enzyme Regul. 43*, 121−134.
2. Kwak, M. K., Wakabayashi, N., and Kensler, T. W. (2004) Chemoprevention through the Keap1−Nrf2 signaling pathway by phase 2 enzyme inducers, *Mutat. Res. 555*, 133−148.
3. Holtzclaw, W. D., Dinkova-Kostova, A. T., and Talalay, P. (2004) Protection against electrophile and oxidative stress by induction of phase 2 genes: The quest for the elusive sensor that responds to inducers, *Adv. Enzyme Regul. 44*, 335−367.
4. Rushmore, T. H., and Pickett, C. B. (1990) Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants, *J. Biol. Chem. 265*, 14648−14653.
5. Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the β-globin locus control region, *Proc. Natl. Acad. Sci. U.S.A. 91*, 9926−9930.
6. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements, *Biochem. Biophys. Res. Commun. 236*, 313−322.
7. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, *Genes Dev. 13*, 76−86.
8. Hayes, J. D., and McMahon, M. (2001) Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention, *Cancer Lett. 174*, 103−113.
9. Kang, M. I., Kobayashi, A., Wakabayashi, N., Kim, S. G., and Yamamoto, M. (2004) Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes, *Proc. Natl. Acad. Sci. U.S.A. 101*, 2046−2051.
10. Motohashi, H., and Yamamoto, M. (2004) Nrf2-Keap1 defines a physiologically important stress response mechanism, *Trends Mol. Med. 10*, 549−557.
11. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T., and Yamamoto, M. (2003) Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles, *Genes Cells 8*, 379−391.
12. McMahon, M., Itoh, K., Yamamoto, M., and Hayes, J. D. (2003) Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression, *J. Biol. Chem. 278*, 21592−21600.
13. Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., and Yamamoto, M. (2004) Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2, *Mol. Cell. Biol. 24*, 7130−7139.
14. Cullinan, S. B., Gordan, J. D., Jin, J., Harper, J. W., and Diehl, J. A. (2004) The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: Oxidative stress sensing by a Cul3-Keap1 ligase, *Mol. Cell. Biol. 24*, 8477−8486.
15. Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J., and Hannink, M. (2004) Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex, *Mol. Cell. Biol. 24*, 10941−10953.
16. Zipper L. M., and Mulcahy, R. T. (2002) The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm, *J. Biol. Chem. 277*, 36544−36552.
17. Li, X., Zhang, D., Hannink, M., and Beamer, L. J. (2004) Crystal structure of the Kelch domain of human Keap1, *J. Biol. Chem. 279*, 54750−54758.
18. Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., and Talalay, P. (2002) Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants, *Proc. Natl. Acad. Sci. U.S.A. 99*, 11908−11913.
19. Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., Kobayashi, A., Yamamoto, M., Kensler, T. W., and Talalay, P. (2004) Protection against electrophile and oxidant stress by induction of the phase 2 response: Fate of cysteines of the Keap1 sensor modified by inducers, *Proc. Natl. Acad. Sci. U.S.A. 101*, 2040−2045.
20. Levonen, A. L., Landar, A., Ramachandran, A., Ceaser, E. K., Dickinson, D. A., Zanoni, G., Morrow, J. D., and Darley-Usmar, V. M. (2004) Cellular mechanisms of redox cell signalling: Role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products, *Biochem. J. 378*, 373−382.
21. Zhang, D. D., and Hannink, M. (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress, *Mol. Cell. Biol. 23*, 8137−8151.
22. Zheng, M., and Storz, G. (2000) Redox sensing by prokaryotic transcription factors, *Biochem. Pharmacol. 59*, 1−6.
23. Paget M. S., and Buttner, M. J. (2003) Thiol-based regulatory switches, *Annu. Rev. Genet. 37*, 91−121.
24. Linke, K., and Jakob, U. (2003) Not every disulfide lasts forever: Disulfide bond formation as a redox switch, *Antioxid. Redox Signaling 5*, 425−434.
25. Giles, N. M., Watts, A. B., Giles, G. I., Fry, F. H., Littlechild, J. A., and Jacob, C. (2003) Metal and redox modulation of cysteine protein function, *Chem. Biol. 10*, 677−693.
26. Jacob, C., Giles, G. I., Giles, N. M., and Sies, H. (2003) Sulfur and selenium: The role of oxidation state in protein structure and function, *Angew. Chem., Int. Ed. 42*, 4742−4758.
27. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
28. Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data, *Anal. Biochem. 182*, 319−326.
29. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage, T4, *Nature 227*, 680−685.
30. Hunt, J. B., Neece, S. H., and Ginsburg, A. (1985) The use of 4-(2-pyridylazo)resorcinol in studies of zinc release from *Escherichia coli* aspartate transcarbamoylase, *Anal. Biochem. 146*, 150−157.
31. Jakob, U., Eser, M., and Bardwell, J. C. (2000) Redox switch of Hsp33 has a novel zinc-binding motif, *J. Biol. Chem. 275*, 38302−38310.
32. Snyder, G. H., Cennerazzo, M. J., Karalis, A. J., and Field, D. (1981) Electrostatic influence of local cysteine environments on disulfide exchange kinetics, *Biochemistry 20*, 6509−6519.
33. Brocklehurst, K. (1979) Specific covalent modification of thiols: Applications in the study of enzymes and other biomolecules, *Int. J. Biochem. 10*, 259−274.
34. Zhou, Z. S., Peariso, K., Penner-Hahn, J. E., and Matthews, R. G. (1999) Identification of the zinc ligands in cobalamin-independent methionine synthase (MetE) from *Escherichia coli*, *Biochemistry 38*, 15915−15926.
35. Berkovits, H. J., and Berg, J. M. (1999) Metal and DNA binding properties of a two-domain fragment of neural zinc finger factor 1, a CCHC-type zinc binding protein, *Biochemistry 38*, 16826−16830.
36. Bertini, I., and Luchinat, C. (1984) High spin cobalt(II) as a probe for the investigation of metalloproteins, *Adv. Inorg. Biochem. 6*, 71−111.
37. Frankel, A. D., Berg, J. M., and Pabo, C. O. (1987) Metal-dependent folding of a single zinc finger from transcription factor IIIA, *Proc. Natl. Acad. Sci. U.S.A. 84*, 4841−4845.

38. Guerrerio, A. L., and Berg, J. M. (2004) Metal ion affinities of the zinc finger domains of the metal responsive element-binding transcription factor-1 (MTF1), *Biochemistry 43*, 5437−5444.
39. Musci, G., and Berliner, L. J. (1985) Probing different conformational states of bovine α-lactalbumin: Fluorescence studies with 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate], *Biochemistry 24*, 3852−3856.
40. Pastukhov, A. V., and Ropson, I. J. (2003) Fluorescent dyes as probes to study lipid-binding proteins, *Proteins 53*, 607−615.
41. Talalay, P., De Long, M. J., and Prochaska, H. J. (1988) Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis, *Proc. Natl. Acad. Sci. U.S.A. 85*, 8261−8265.
42. Spencer, S. R., Xue, L. A., Klenz, E. M., and Talalay, P. (1991) The potency of inducers of NAD(P)H:(quinone-acceptor) oxidoreductase parallels their efficiency as substrates for glutathione transferases. Structural and electronic correlations, *Biochem. J. 273*, 711−717.
43. Prestera, T., Zhang, Y., Spencer, S. R., Wilczak, C. A., and Talalay, P. (1993) The electrophile counterattack response: Protection against neoplasia and toxicity, *Adv. Enzyme Regul. 33*, 281−296.
44. Dinkova-Kostova, A. T., Massiah, M. A., Bozak, R. E., Hicks, R. J., and Talalay, P. (2001) Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups, *Proc. Natl. Acad. Sci. U.S.A. 98*, 3404−3409.
45. Graf, P. C., Martinez-Yamout, M., VanHaerents, S., Lilie, H., Dyson, H. J., and Jakob, U. (2004) Activation of the redox-regulated chaperone Hsp33 by domain unfolding, *J. Biol. Chem. 279*, 20529−20538.
46. Li, W., Bottrill, A. R., Bibb, M. J., Buttner, M. J., Paget, M. S., and Kleanthous, C. (2003) The role of zinc in the disulphide stress-regulated anti-sigma factor RsrA from *Streptomyces coelicolor*, *J. Mol. Biol. 333*, 461−472.
47. Bae, J. B., Park, J. H., Hahn, M. Y., Kim, M. S., and Roe, J. H. (2004) Redox-dependent changes in RsrA, an anti-sigma factor in *Streptomyces coelicolor*: Zinc release and disulfide bond formation, *J. Mol. Biol. 335*, 425−435.
48. Maret, W. (2004) Zinc and sulfur: A critical biological partnership, *Biochemistry 43*, 3301−3309.

BI047434H