Reduction of Phenoxyl Radicals by Thioredoxin Results in Selective Oxidation of Its SH-Groups to Disulfides. An Antioxidant Function of Thioredoxin†

Radoslav Goldman,‡ Detcho A. Stoyanovsky,‡ Billy W. Day,‡,§,|| and Valerian E. Kagan*,‡,§

Department of Environmental and Occupational Health and Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, and Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15238

Received November 11, 1994; Revised Manuscript Received January 17, 1995⊗

ABSTRACT: Thioredoxin is an important cellular redox buffer. In this report, we describe the reaction of thioredoxin with phenoxyl radicals. The vicinal sulfhydryls of the bis(cysteinyl) active site sequence reduced phenoxyl radicals released in horseradish peroxidase-catalyzed oxidation of phenol. Redox cycling of phenol was accompanied by selective oxidation of thioredoxin sulfhydryls to disulfides. HPLC/UV−vis measurements showed that the SH:phenol oxidation ratio was 15:1 under the conditions used. At the end of the reaction, oxidized thioredoxin was quantitatively recovered in the reduced form with dithiothreitol. Oxidation of sulfhydryls to sulfoxy derivatives, oxidation of other amino acid residues, and formation of covalent adducts with phenolic metabolites (quinones) were not detected by LC−MS. While the thiyl radical of glutathione was readily detected with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide, no ESR-detectable DMPO−thiyl adducts formed during the oxidation of thioredoxin. Similarly, oxidation of vicinal sulfhydryls of dihydrolipoic acid did not produce DMPO−thiyl spin adducts, indicating that fast intramolecular cyclization to disulfide occurred with thioredoxin. Measurements of the superoxide dismutase-sensitive chemiluminescence response of lucigenin demonstrated that thioredoxin oxidation was accompanied by release of superoxide, most likely via disulfide radical anion-mediated one-electron reduction of oxygen. We propose that formation of disulfides is characteristic of the phenoxyl radical-catalyzed oxidation of vicinal sulfhydryls in both small thiols and disulfide-forming oxidoreductases. Reversibility of the phenoxyl radical-catalyzed modification of thioredoxin may be responsible for its function as an efficient cytosolic antioxidant.

The reducing potential of cytoplasm is substantially greater than that of the extracellular environment or the lumen of the endoplasmic reticulum (Derman et al., 1993). This is mainly due to two ubiquitous cytosolic redox buffers, glutathione (GSH),[1] and thioredoxin (TRX) (Gilbert, 1990), whose reduced states are maintained by their respective NADPH-dependent reductases, glutathione reductase and thioredoxin reductase (Williams, 1976; Moore et al., 1964). Both buffers maintain the reducing environment of the cytoplasm through thiol−disulfide exchange reactions (Gilbert, 1990). The redox potentials of both redox couples are comparable ($E^{\circ\prime}$= −260 mV for GSSG/GSH; $E^{\circ\prime}$= −270 mV for TRX(S−S)/TRX(SH))[2] (Holmgren, 1985), and the similarity of the modular structure of the nucleotide-binding domains of the GSH and TRX reductases shows that they have evolved from a common ancestor (Kuriyan, 1993). Yet, the cellular functions of the redox buffers are complementary.

GSH, a cysteine-containing tripeptide, is the most abundant thiol in mammalian cells (Meister, 1983). GSH plays an important role in amino acid transport, protein synthesis, detoxification of xenobiotic compounds, and protection against reactive oxygen species and free radicals (Gilbert, 1990; Bray & Taylor, 1993).

The ubiquitous isoforms of TRX are found in all eukaryotic and prokaryotic cells at low micromolar concentrations (Holmgren, 1978, 1985). These 12-kDa proteins share the catalytic site conserved sequence X-Cys-X-X-Cys with the family of thiol protein oxidoreductases (Claiborne et al., 1992). The two cysteinyl sulfhydryls form a 14-member disulfide-containing ring upon oxidation (Eklund et al., 1991). Intramolecular cyclization is the major difference between the oxidation of TRX and GSH, which forms its disulfides only in bimolecular reactions. The reduction potential of thioredoxins reflects the propensity of the bis(cysteinyl) sequence to form the disulfide loop (Siedler et al., 1993). This, in turn, is reflected in their ability to reduce disulfide bonds in various proteins. The absence of disulfide bonds in cytoplasmic proteins and their presence in exported proteins is generally associated with the activity of the TRX/TRX reductase system (Derman et al., 1993). TRX reacts with a broad range of substrates including insulin (Holmgren, 1979), ribonucleotide reductase (Thelander & Reichard, 1979), neurotoxins, a broad class of low molecular weight proteins, (Lozano et al., 1994), and tetanus toxin (Kistner et al., 1993). Reduction of disulfide bonds in proteins is therefore the major function of TRX in non-photosynthetic organisms. GSH cannot substitute for TRX in the majority of these reactions (Holmgren 1989).

† This work was supported by Grant 94A45 from the American Institute for Cancer Research.

* Correspondence should be addressed to this author at Department of Environmental and Occupational Health, University of Pittsburgh, Kappa Drive, RIDC Park, Pittsburgh, PA 15238. Telephone (412) 967-6516. Fax: (412) 624-1020.

‡ Department of Environmental and Occupational Health, University of Pittsburgh.

§ Pittsburgh Cancer Institute.

|| Department of Pharmaceutical Sciences, University of Pittsburgh.

⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1995.

[1] Abbreviations: GSH, glutathione; DHLA, dihydrolipoic acid; TRX, thioredoxin; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; HRP, horseradish peroxidase; SOD, superoxide dismutase; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; NHE, normal hydrogen electrode.

[2] Standard potential vs NHE at pH 7.0 and 25 °C.

0006-2960/95/0434-4765$09.00/0

While the reduction of free radicals is usually associated with GSH, several facts indicate that TRX may complement this function. It was shown that the null thioredoxin−glutaredoxin double mutant of *Escherichia coli* is viable only under semi-anaerobic conditions, indicating an active role of TRX in the response to oxidative stress (Miranda-Vizuete et al., 1994). The concentration of reduced GSH decreases dramatically in cells exposed to oxidative stress (Cadenas & Sies, 1985; Chance et al., 1979). Protein sulfhydryls are the fastest-reacting antioxidant component in plasma (Wayner et al., 1987), and TRX may be kinetically a better reductant of radicals than GSH (vide infra).

Our ongoing studies of the metabolism of phenolic compounds prompted us to investigate the reaction of phenoxyl radicals with thiols. Peroxidase-catalyzed release of phenoxyl radicals is a well-characterized reaction (Sakurada et al., 1990; Baek & Van Wart, 1992) with important toxicological ramifications related to the bioactivation of phenolic metabolites of benzene (Sabourin et al., 1992, and references therein) and the metabolism of phenolic drugs (Rao et al., 1990; Aust et al., 1993). Reduction of phenoxyl radicals by low M_r thiols, including GSH, has been well-described by several groups (Surdhar & Armstrong, 1986; Asmus, 1990; Mason & Rao, 1990). The resulting GSH thiyl radical may lead to enzyme inhibition, mutagenesis, and oxidation of DNA and fatty acids because of its reactivity (Yim et al., 1994, and references therein). This suggests that GSH is not an ideal reductant of radical intermediates. We have shown previously that phenoxyl radicals oxidize protein thiols and GSH in both model systems and in cell and tissue homogenates (Kagan et al., 1994; Tyurina et al., 1994). We have therefore begun a systematic characterization of the reaction of the phenoxyl radical of phenol with proteins. On the basis of the differences in the reactivity of low-M_r thiols (Mason & Rao, 1990), we have divided proteins into two broad groups for our purposes: (1) disulfide-forming proteins and (2) proteins with isolated cysteines. TRX has ideal properties for the characterization of the phenoxyl radical-catalyzed modification of disulfide-forming proteins.

In this report, we describe the reaction of *E. coli* TRX with the phenoxyl radical of phenol. To characterize the reaction mechanism of phenoxyl radicals with TRX, we quantified the oxidation of TRX thiols in parallel with reverse-phase HPLC measurements of phenol and its oxidation products. We measured concurrently modification of TRX by pneumatically assisted electrospray mass spectrometry (LC−MS). We also attempted to detect the intermediates of thiol oxidation, thiyl radicals and disulfide radical anions, by electron spin resonance (ESR) and to detect the release of superoxide by spin trapping and by monitoring lucigenin-mediated chemiluminescence.

MATERIALS AND METHODS

Materials

E. coli TRX was purchased from Promega (Madison, WI); Chelex-100 was from Bio-Rad (Hercules, CA); and methanol, ethanol, acetonitrile, and acetic acid from Fisher Scientific (New Lawn, NJ). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Water was distilled in quartz glassware. Aqueous buffers and solutions were treated with Chelex-100 prior to all experiments.

Methods

Preparation of TRX. *E. coli* TRX was reduced with 10-fold excess dithiothreitol (DTT) at 27 °C for 30 min prior to all experiments. The reduced protein was extensively dialyzed against Chelex-100-treated ddH_2O saturated with nitrogen. Under these conditions, the protein was fully reduced (2 SH groups/molecule) and the recovery (based on thiol titration and protein absorbance) was nearly quantitative (Kallis & Holmgren, 1980). For experiments on the recovery of oxidized TRX, the protein and DTT were separated on a PD-10 Sephadex G-25M column, 9-mL bed volume (Pharmacia, Uppsala, Sweden), equilibrated with 0.1 M phosphate buffer saturated with sodium chloride. The protein, eluting within the first 2 mL, was well-separated from DTT, and it was immediately reacted with dithionitrobenzoic acid (DTNB).

HPLC−UV−Vis Measurements. All spectrophotometric measurements were carried out using a UV160U spectrophotometer (Shimadzu, Kyoto) with a constant-temperature controller. The concentration of thiols was determined with DTNB using Ellman's procedure (Ellman, 1959). A standard curve was established by additions of GSH (10−100 μM) to DTNB solution (200 μM) in phosphate buffer (10 mM, pH 7.4). The DTNB blank was subtracted in the reference beam and absorbance was measured at 412 nm using an ϵ of 13.6 mM^{-1} cm^{-1}.

Reaction Conditions. A typical oxidation reaction mixture contained HRP type VI-A, RZ 3.0 (0.25 unit/mL), phenol (160 μM), H_2O_2 (150 μM), and thiol (TRX, GSH, DHLA, 50 μM to 1.5 mM) in 50 mM phosphate buffer, pH 7.4. The reaction was carried out at 25 °C and was stopped with catalase (300 units/mL) when required. For HPLC measurement of phenols, 50 μL aliquots of the reaction mixture were withdrawn at given time intervals and the reaction was stopped with 0.2 mL of 5% trichloroacetic acid (TCA) in ethanol. The resulting solution was filtered through a 1-mL C18 Sep Pak cartridge (Waters Division of Millipore Co., Milford, MA), and the filtrates were analyzed by HPLC. A Shimadzu LC-10A system equipped with an LC-600 pump and an M10A photodiode-array detector was used for separations of the phenol oxidation mixture. A C18 reverse-phase column (Ultrasphere ODS, 5 μm particle size, 4.6 × 250 mm, Beckman) was used with a mobile phase of 50% aqueous CH_3OH adjusted to pH 3.1 with CH_3COOH at a flow rate of 1.5 mL/min. Under these conditions, the retention time for phenol was 3.62 min and its oxidation products were well-separated as determined by the sequential UV-vis spectra of the peaks.

Chemiluminescence Measurements. Superoxide anions were monitored by lucigenin-dependent chemiluminescence (Faulkner & Fridovich, 1993). The reaction was carried out in 1.0 mL of 0.1 M phosphate buffer, pH 7.4, containing lucigenin (50 μM), HRP (0.8 unit/mL), phenol (160 μM), H_2O_2 (300 μM), and thiol (TRX, DHLA, 600 μM) using a Coral Biomedical 633 chemiluminescence analyzer (San Diego, CA) equipped with a BioOrbit dispenser. The chemiluminescence responses were recorded on a chart recorder (WR 3101, Graphtec, Inc., Japan). The records were transferred to an IBM compatible personal computer and analyzed as PCX files.

ESR Measurements. Thiyl radicals were detected as 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) adducts. ESR mea-

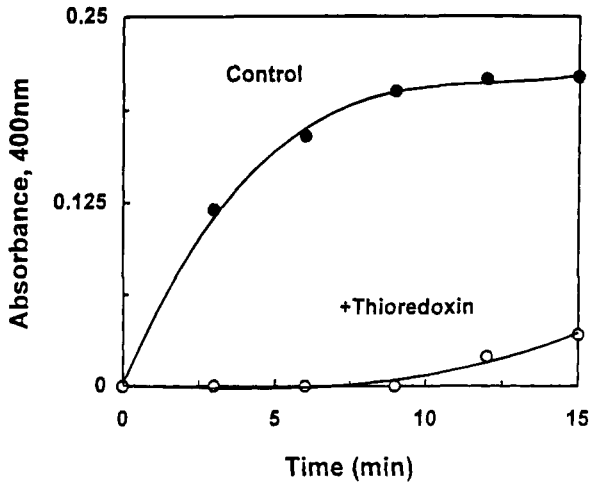


FIGURE 1: Time course of the buildup of phenol polymers. Control (●): HRP, 0.25 unit/mL; H_2O_2, 150 μM; phenol, 160 μM; phosphate buffer, 50 mM, pH 7.4 at 25 °C in 1 mL total volume. Thioredoxin (○): same as control with thioredoxin, 50 μM.

surements were performed on a JEOL-RE1X spectrometer at 25 °C in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.0013 mm thickness, Alpha Wire Co., Elizabeth, NJ). The tubing was filled with 60 μL of sample, folded into quarters, and placed in an open ESR quartz tube in such a way that all the sample was within the effective microwave irradiation area. Spectra were recorded at 3355 G center field, 20 mW power, 0.5−2.0 G modulation, and 25 G/min scan time. Spectra were collected using the EPRWare software (Scientific Software Services, Bloomington, IL) and transferred as ASCII files to the EPR analysis software from Free Radical Metabolite Research Group (NIEHS, Research Triangle Park, NC) for spectral simulations. The GSH thiyl radicals were detected under standard conditions with 100 mM DMPO and 50 μM to 1 mM GSH. For the detection of superoxide and hydroxyl radical adducts, DMPO was cleaned with charcoal from its oxidation products and 0.3 mM EDTA was added to the phosphate buffer. We have used DHLA (1.2 mM), phenol (0.5 mM), H_2O_2 (0.7 mM), and HRP (0.3−0.8 unit/mL) or TRX (1.2 mM) under identical conditions.

Mass Spectrometric Measurements. Mass spectrometric determinations of TRX were carried out on a Perkin-Elmer/Sciex API 1 spectrometer equipped with an atmospheric pressure ionization source and an IonSpray interface which was maintained at 5 kV. The orifice was maintained at 70 V. High-purity N_2 flowing at 0.6 L/min served as the curtain gas, and high-purity air maintained at 40 psi was used for nebulization. Samples were introduced using a Hewlett-Packard 1090 Series II liquid chromatograph/diode-array 1040A detector, without column separation (contamination with HRP was negligible) and without splitting of the effluent. Typically, 2 nmol of protein was injected, either directly from the reaction mixture or after dialysis against ddH_2O, using 50% aqueous CH_3CN with 0.05% trifluoroacetic acid as the solvent flowing into the ionization source at 40 μL/min. Mass spectra were acquired every 6−12 s over the range of *m/z* 1000−2400 (0.1 *m/z* resolution). Analytes were detected as their $[M + H]^+$, $[M + K]^+$, or $[M + Na]^+$ ions.

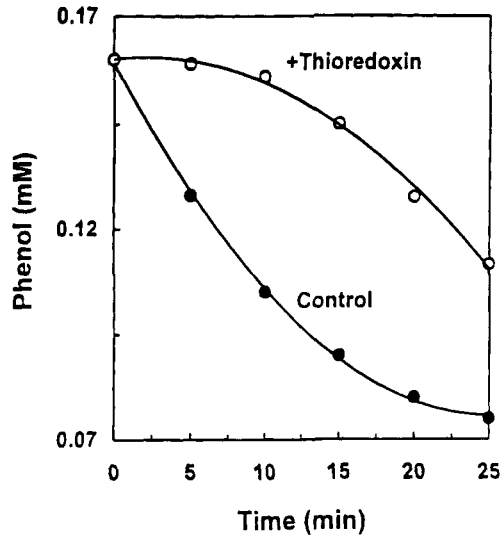
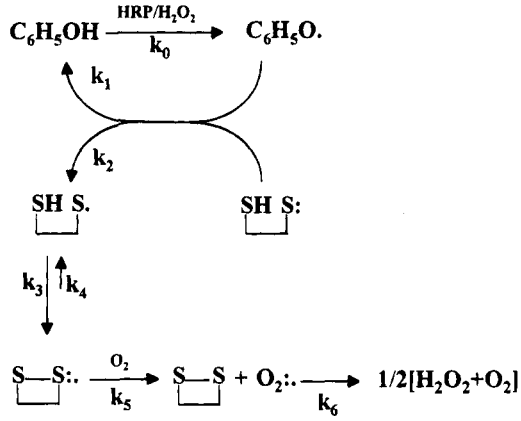


FIGURE 2: Time course of phenol oxidation. Control (●): HRP, 0.25 unit/mL; H_2O_2, 150 μM; phenol, 160 μM; phospate buffer, 50 mM, pH 7.4 at 25 °C in 1 mL total volume. Thioredoxin (○) : control with thioredoxin, 50 μM.

Scheme 1



RESULTS

Phenoxyl Radical-Catalyzed Oxidation of TRX. The formation of polymers absorbing at 400 nm is characteristic of the peroxidative metabolism of phenol (Moore et al., 1992). In the presence of TRX, the absorbance of phenol polymers did not appear until TRX was oxidized (Figure 1). HPLC measurements showed that the initial rate of phenol oxidation (k_o, Scheme 1; assume standard concentrations for all rates as discussed below) was 4 nmol/min (Figure 2). With TRX added, the phenol oxidation proceeded at a rate of only 0.5 nmol/min [$(k_o - k_1)$, Scheme 1] during the first 10 min and resumed at the rate of the control (without TRX) at the 15th minute (Figure 2). The rate of phenol oxidation was not changed by the addition of oxidized TRX.

Oxidation of TRX sulfhydryls was slow without addition of phenol (1.0 nmol/min). The rate of oxidation increased about 7-fold with the addition of phenol (k_2 = 7.5 nmol of SH/min, Scheme 1) such that the oxidation was complete within 15 min of incubation (Figure 3). On the basis of these measurements, we can estimate that for each phenol oxidized to products 15 TRX SH groups were oxidized (Figures 2 and 3). This clearly indicates recycling of phenol from its phenoxyl radical.

Recovery of Reduced Form of TRX with DTT. At the end of reaction with HRP/H_2O_2/phenol, TRX did not contain any

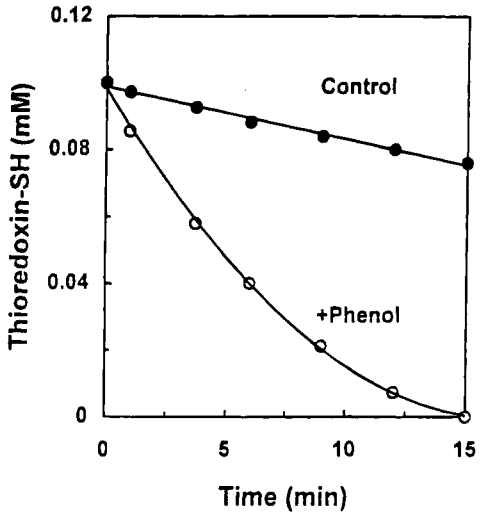


FIGURE 3: Time course of thioredoxin oxidation. Control (●): without phenol, conditions as in Figure 2 (same sample). Phenol (○): control with phenol, 160 μM. Reaction was stopped with catalase in 50-μL aliquots of the reaction mixture.

Table 1: Recovery of Thioredoxin Sulfhydryls with DTT after Phenoxyl Radical-Catalyzed Oxidation

	TRX-SH (μM)	TRX-SH (% of control)
before oxidation	140	100
after oxidation	0	0
after oxidation + DTT	132	94

DTNB-titratable sulfhydryls (Table 1). Reduction of the oxidized TRX with excess DTT resulted in regeneration of ~94% of its sulfhydryl groups.

Mass Spectrometric Measurements of Oxidized TRX. The size of thioredoxin allows direct molecular weight determination by electrospray mass spectrometry without proteolytic cleavage of the protein (Winger et al., 1993). The mass spectrum of fully oxidized TRX (the same sample that was used for phenol and SH measurements) was compared with the spectrum of fully reduced TRX. The positive ion spectra, with the typical multiply charged ion envelopes, and the reconstructed mass spectra (Fenn method) of oxidized and reduced thioredoxin were identical (Figure 4). Except for the usual $[M + H]^+$, $[M + K]^+$, or $[M + Na]^+$ quasi-molecular ions, no changes in molecular mass of thioredoxin were detectable (Figures 4A and 5A). The measured molecular mass of TRX corresponds to the calculated mass of 11 673 amu based on the known sequence (Eklund et al., 1991) within the 3−5-amu resolution of the method. We searched specifically for oxygen adducts of the two thioredoxin cysteines. We could not detect any sulfoxy derivatives. Furthermore, the mass of the sample did not change between the 15th and 25th min of reaction (after complete oxidation of the sulfhydryl groups), so that further oxidation of TRX at residues other than cysteines did not occur. We were, however, able to detect almost quantitative covalent binding of two quinones per TRX (modified molecular mass, 11 889 amu) when the reduced protein was reacted with a 4-fold excess of *p*-quinone at 25 °C for 2 h (Figure 5B).

ESR Measurements of Radical−DMPO Spin Adducts. A typical ESR signal of the GSH−DMPO thiyl radical adduct (Mason & Rao, 1990) was detected when phenol/HRP/H_2O_2 were reacted with GSH (Figure 6); omitting any one component prevented adduct formation under the conditions used. In contrast to the GSH thiyl radical, we could not trap the TRX-derived thiyl radicals under the same conditions. Likewise, DHLA did not produce detectable DMPO adducts upon incubation with phenol/HRP/H_2O_2. At higher

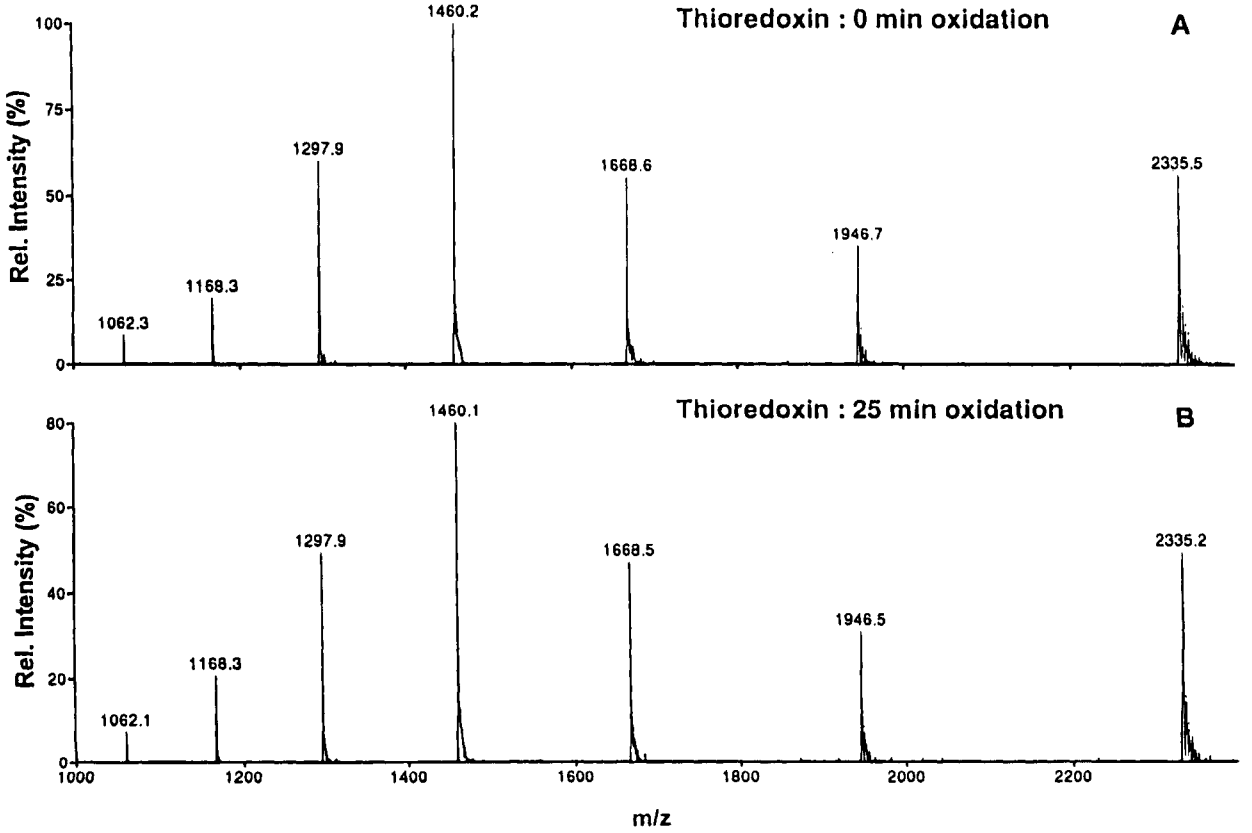


FIGURE 4: Mass spectrometric measurements of thioredoxin. Oxidation was carried out as described in Figure 2 (same sample). The spectra of aliquots withdrawn at 0, 15, and 25 min were identical. (A) Sample withdrawn at 0 min of oxidation. (B) Sample withdrawn at 25 min of oxidation.

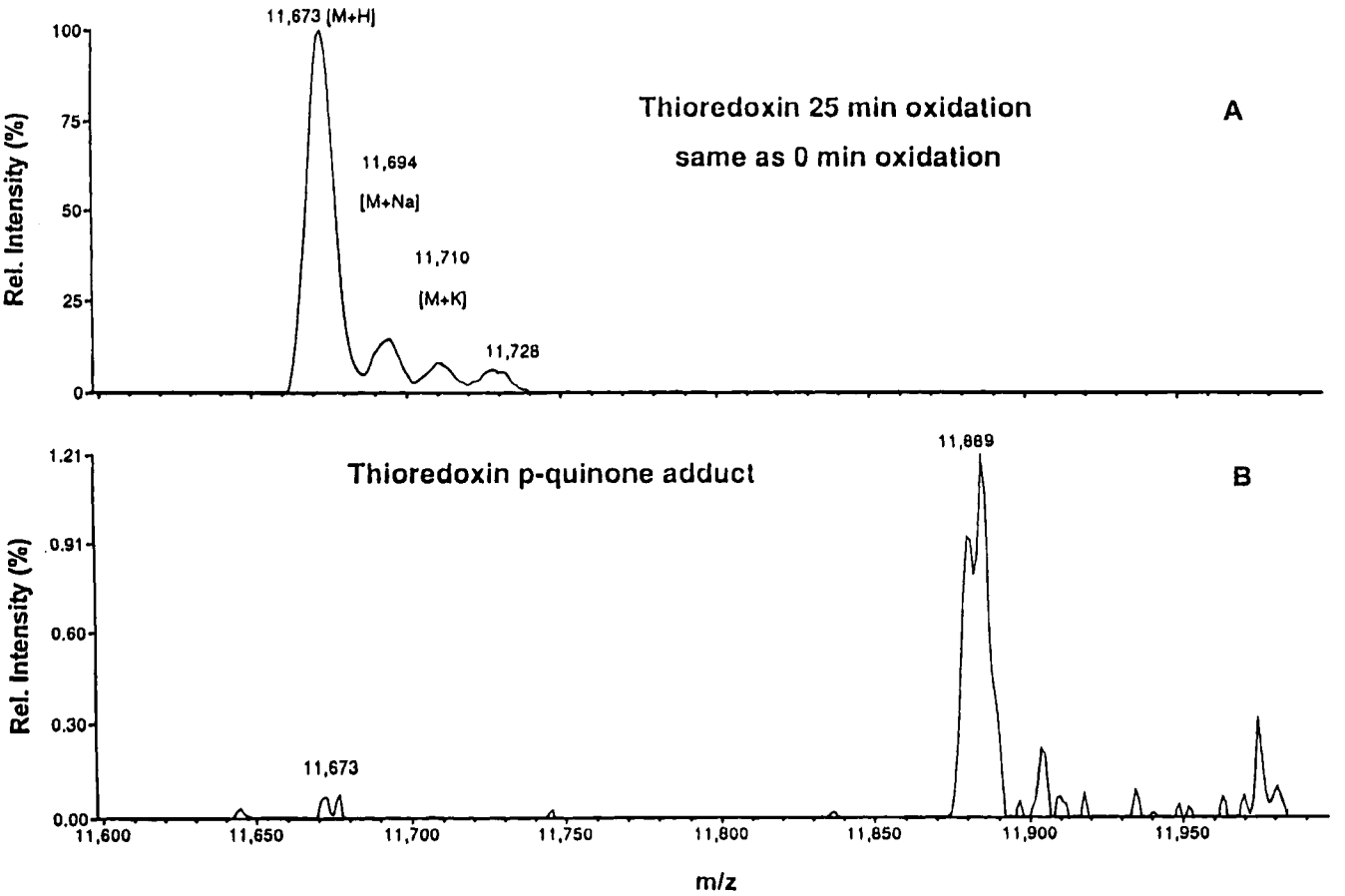


FIGURE 5: Reconstruction of the molecular mass. The calculated mass (based on the sequence of thioredoxin) is 11 673 Da. (A) The $[M + H]^+$, $[M + K]^+$, and $[M + Na]^+$ ions are present in samples of both oxidized and reduced thioredoxin. (B) The mass of *p*-quinone–thioredoxin adduct, 11 889 Da, corresponds to 2 quinones/thioredoxin adduct and is well within the 5-amu resolution of the method. Native thioredoxin is almost absent. Conditions are described in Materials and Methods.

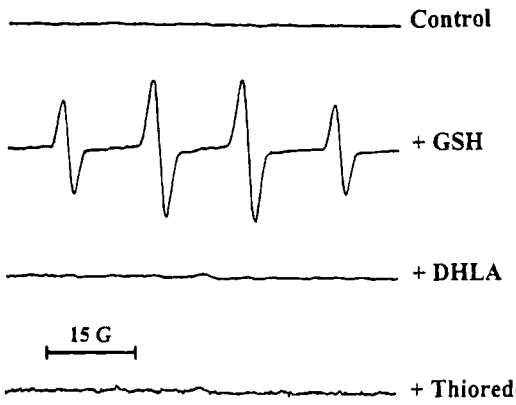


FIGURE 6: ESR measurements of thiyl radical–DMPO adducts. Control: Phenol/HRP/H_2O_2 and DMPO as described in Materials and Methods. Thiols (100 μM) were added as indicated. The GSH thiyl radical adduct has splitting constants a^N = 15.4 G and a^b = 16.2 G.

concentrations of DMPO and other components, the superposition of superoxide and hydroxyl radical ESR signals was detected; an initial 8:1 ratio slowly shifted to more DMPO/OH• over a 20-min period (Figure 7). When HRP/H_2O_2 oxidized DHLA directly in the absence of phenol, only the DMPO/OH• signal appeared in the ESR spectrum. Only traces of unresolved signal were detected when DHLA was replaced with TRX under comparable conditions (Figure 7).

Lucigenin-Mediated Detection of Superoxide by Chemiluminescence. To form the disulfide, the postulated TRX disulfide radical anion must reduce an electron acceptor, e.g., oxygen (see Scheme 1), as has been previously shown with other thiols (Asmus, 1989; Mason & Rao, 1990). Using lucigenin-sensitized chemiluminescence (Faulkner & Fridovich, 1993), we detected superoxide production in the reaction of TRX with phenol/HRP/H_2O_2 (Figure 8). The chemiluminescence response was SOD-sensitive and thiol-dependent. Chemiluminescence was not detected without phenol or HRP, or both, and only traces of signal were detected with HRP/H_2O_2/phenol when TRX was omitted. A more intense SOD-quenchable chemiluminescence response was observed with DHLA (Figure 8).

DISCUSSION

Our experiments describe for the first time the reaction of the phenoxyl radical of phenol with TRX. While the reactions of proteins with indiscriminately reactive oxygen radicals (e.g., hydroxyl and alkoxyl radicals) are, in general, destructive events marking proteins for proteolytic degradation (Pacifici et al., 1989; Stadtman, 1993), the reaction with phenoxyl radicals seems to be selective and depends largely on their redox potential. Phenoxyl radicals derived from some natural phenolic compounds, e.g., α-tocopherol (vitamin E), have very low reactivity toward thiols (Niki, 1982). Reports in the literature and our previous results indicate that phenoxyl radicals of other phenols (e.g., phenol, *p*-cresol, dimethoxyphenol, etoposide) readily oxidize thiols (Katki et al., 1987; Schreiber et al., 1989; Kagan et al., 1994) and potentially other amino acid residues (Brabec & Mornstein, 1980). On the basis of the chemical precedent with low M_r thiols, we hypothesize that the vicinal sulfhydryls of TRX will react according to Scheme 1.

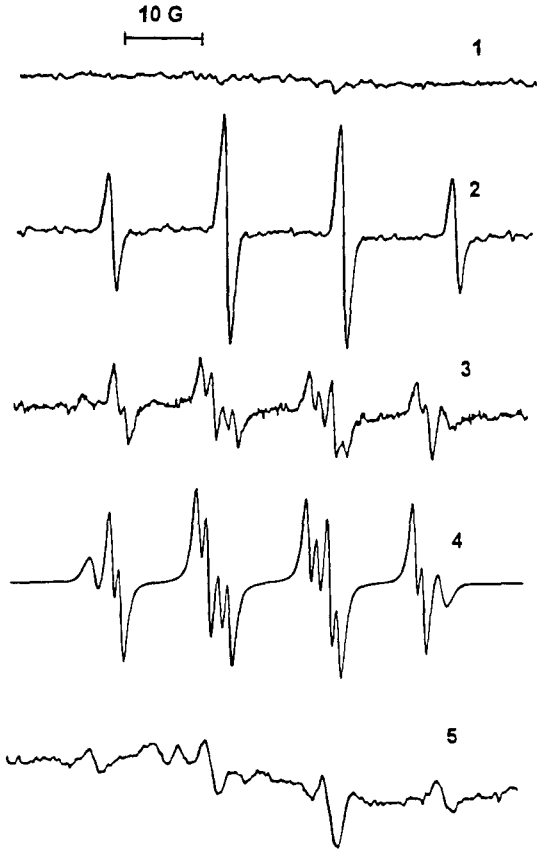


FIGURE 7: ESR measurements of oxyradical−DMPO adducts. (1) Phenol/thiol/H_2O_2 and DMPO as specified in Materials and Methods. (2) HRP/DHLA/H_2O_2 and DMPO. (3) Phenol/HRP/DHLA/H_2O_2 and DMPO. (4) Simulation of spectrum 3 using a 9:1 ratio of DMPO/OOH• (a^N = 14.1 G; a^{H1} = 11.4 G; a^{H2} = 1.2 G; 90% Lorentzian content, 0.6 G line width) to DMPO/OH• (a^N = 14.9 G; a^H = 14.9 G; 25% Lorentzian content, 0.6 G line width). (5) Phenol/HRP/H_2O_2/TRX and DMPO.

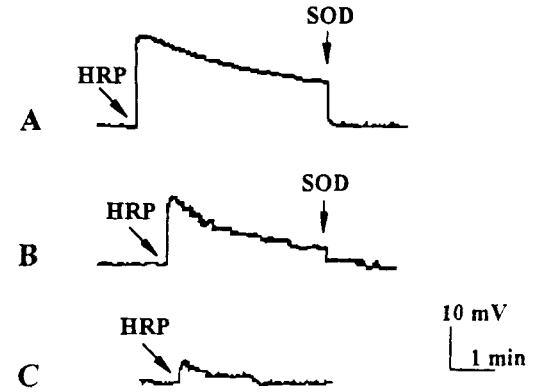


FIGURE 8: Chemiluminescence measurements of superoxide. (A) Phenol/HRP/H_2O_2 and DHLA. (B) Phenol/HRP/H_2O_2 and thioredoxin. (C) Phenol/HRP/H_2O_2 without thiols. Conditions are described in Materials and Methods.

Possible alternative reactions include oxygenation of the cysteines, oxidation of other amino acid residues, intramolecular long-range electron transfer, and binding of the phenol oxidation products (quinones). We therefore designed experiments to determine the reaction mechanism with the emphasis on the physiological reversibility of phenoxyl radical-induced oxidative modification of TRX. For comparison, we have studied the interaction of phenoxyl radicals with low M_r thiols, GSH and DHLA. We will follow the summary of our results with the outline of some relevant points connecting them with the mechanism of toxicity of phenolic compounds.

Redox Cycling of Phenol by TRX. TRX is a good reductant with a two-electron reduction potential of −0.27 V. Does the protein readily undergo one-electron oxidations? We can compare the oxidation of TRX with that of the small thiols (GSH, DTT, dihydrolipoamide) whose reactions with the phenoxyl radical of phenol were described (Surdhar & Armstrong, 1986; Rao et al., 1990). The reduction potentials of the GSH [S•/SH] and DTT [S−S•⁻/SH] couples at pH 7.4, estimated by the published equations, are appropriate for phenoxyl radical reduction in both cases (E[GS•/GSH] = 0.89 V and E[DS−S•⁻/DSH] = 0.84 V; E[PhO•/PhOH] = 0.94 V, all at pH 7.4) (Lind et al., 1990).

The active site cysteines of *E. coli* TRX, Cys32 and Cys35, have low pK_a values (7.1 and 7.9) (Dyson et al., 1991). Deprotonation of the cysteines at physiological pH facilitates one-electron oxidations to thiyl radicals. The negative charge stabilizes the [S S•] radical formed, and fast intramolecular ring closure to disulfide radical anion, which reduces O_2, forces the equillibrium toward thiol oxidation (Asmus, 1990). It is therefore likely that the reduction potentials for the one electron oxidation of thioredoxin will be even more favorable (less positive) than is the case for GSH. In addition, results obtained by cyclic voltammetry indicate that two quasi-reversible one-electron steps for the oxidation of thioredoxin exist (Salamon et al., 1992). This indicates that the reduction of phenoxyl radicals by thioredoxin is plausible.

Our experiments showed that the phenoxyl radical of phenol was indeed reduced by thioredoxin. It is not surprising that vicinal sulfhydryls reduce the phenoxyl radical of phenol. The efficiency of the redox cycling by a protein (15 SH-groups oxidized per phenol converted to oxidation products), however, was unexpected. The rates of the individual steps certainly deserve further study.

Radical Intermediates in the Reaction Mechanism. Thiyl radical and disulfide radical anion are the expected intermediates of the radical-catalyzed oxidation of TRX−cysteines to disulfide (Scheme 1). In contrast to the GSH thiyl radicals, the thioredoxin-derived thiyl radicals could not be trapped under the same conditions. Likewise, DHLA did not produce detectable DMPO adducts upon incubation with phenol/HRP/H_2O_2, which agrees with results of other investigators (Cadenas, 1992) (Figure 6). This indicated that the formation of the intramolecular disulfide was faster than the reaction of the radical with DMPO. Our attempts to detect the disulfide radical anion were also unsuccessful.

Support for the proposed reaction mechanism comes from the measurements of reaction rates, the pattern of protein oxidation products, the formation of oxygen-centered DMPO spin adducts, and the detection of lucigenin-mediated superoxide chemiluminescence. TRX was oxidized at a rate of 7.5 nmol of SH/min by HRP/H_2O_2/phenol (see Figure 3). In contrast, 4 nmol/min of phenol was oxidized without TRX in the reaction mixture (see Figure 2); the ratio is 1.88 SH groups oxidized per each phenoxyl radical formed. Oxidation of nearly two SH groups by each phenoxyl radical formed is consistent with the proposed formation of an intramolecular disulfide bond by thiyl radical/thiolate anion coupling (assuming that the rate of phenol oxidation does not change with the addition of thioredoxin).

The signal of DMPO spin adducts detected by ESR with high concentrations of DHLA corresponded to the superposition of DMPO/OOH• and DMPO/OH• signals (Figure 7).

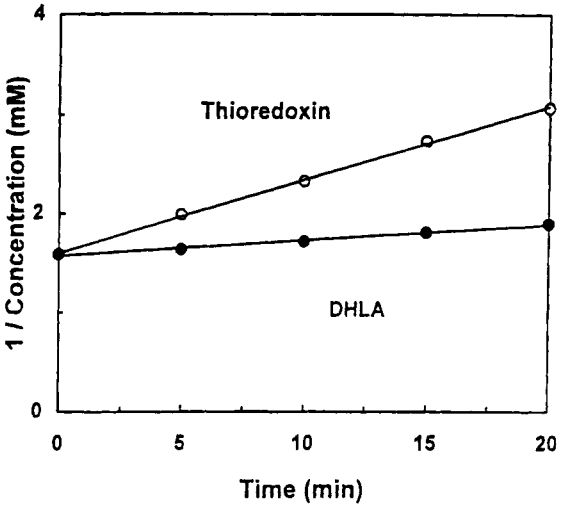


FIGURE 9: Plot of the second-order kinetics of thiol oxidation by hydrogen peroxide. Thiol [TRX (○) 0.6 mM, or DHLA (●) 0.6 mM] was reacted with H_2O_2, 0.6 mM in phosphate buffer, pH 7.4 at 25 °C.

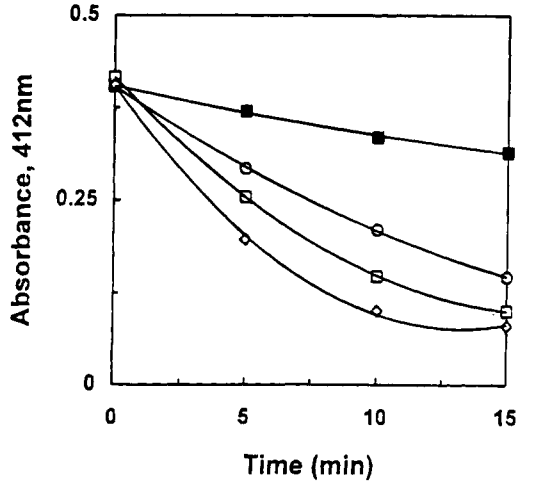


FIGURE 10: Dependence of the reaction rate on HRP concentration. (■) Control, H_2O_2, 500 μM; phenol, 170 μM; TRX, 500 μM; phospate buffer, 50 mM, pH 7.4 at 25 °C. (○) +HRP, 0.1 unit/mL. (□) +HRP, 0.3 unit/mL. (◆) +HRP, 0.5 unit/mL.

DMPO/OOH• was the dominant component when phenol was present and was slowly converted to DMPO/OH• signals by peroxidase-catalyzed decomposition of the adduct. Only the DMPO/OH• signal appeared when phenol was absent. It is likely that phenol competes with the DMPO adduct for peroxidase (Kalyanaraman, 1982). The signals were dependent on thiol concentration and could not be detected at DHLA concentrations below 1 mM. Only traces of unresolved signal were detected with TRX. This was likely due to the competition of the direct bimolecular oxidation of TRX by hydrogen peroxide with the peroxidase-catalyzed oxidation via phenoxyl radicals (Figure 9) (Little & O'Brien, 1969; Coan et al., 1992). The bimolecular reaction was much faster with TRX (k_{obs} = 69 M^{-1} min^{-1}), whose deprotonation to thiolate anion enhanced its nucleophilic attack on hydrogen peroxide, than with DHLA (k_{obs} = 16 M^{-1} min^{-1}), whose reaction was limited to the surface of micelles. The HRP-catalyzed oxidation dominated at low TRX concentrations and was proportional to the HRP concentration (Figure 10). The effective lowering of concentration by hydrogen peroxide prevented detection of resolved oxyradical adducts in the case of TRX. It is also possible that reaction of thioredoxin with superoxide, analogous to the one reported for DTT (Zhang et al., 1991), prevented detection of resolved superoxide signal.

Further evidence for the proposed mechanism came from the chemiluminescence measurements. Higher sensitivity of the method enabled us to use lower concentrations of thiols and H_2O_2. We detected thiol-dependent and SOD-sensitive superoxide production in the reaction of phenol with HRP and H_2O_2 with both DHLA and TRX (Figure 8). It is likely that oxygen was reduced by the disulfide radical anion. Only traces of chemiluminescence (due to the reactions of phenol oxidation products with oxygen) were detected when thioredoxin and DHLA were omitted from the reaction.

Selective Cysteine/Disulfide Conversion of TRX. Almost full recovery of reduced TRX with DTT (Table 1) indicated that cysteines were oxidized to disulfides. Mass spectrometric measurements of oxidized thioredoxin confirmed the selective oxidation of cysteine to cystine. The increases of molecular mass expected in the case of sulfoxidation or formation of quinone adducts were not detectable. Not only were the cysteines converted selectively to disulfides, but also amino acids other than cysteine did not react with the phenoxyl radicals. Literature reports indicate that aromatic amino acids (Y, W, H) and methionine could be oxidized (Brabec & Mornstein, 1980; DeFelippis et al., 1989). Models from crystallographic studies of TRX (Holmgren et al., 1975; Katti et al., 1990) and high-resolution NMR solution studies of both oxidized and reduced TRX (Jeng et al., 1994) show that tyrosine, tryptophan, and methionine residues are on the surface of the protein and accessible to oxidizing species. The formation of only one oxidation product, TRX disulfide, in this complex reaction mixture was somewhat unexpected. It thus seems that the redox potential of the non-cysteine TRX residues is higher than the redox potential of the phenoxyl radical/phenol couple, and there is no favorable reaction to displace the equilibrium to their oxidation, as in the case of the cysteines.

Physiological and Pathophysiological Relevance The measurements showed that TRX disulfide was the major product in phenoxyl radical-catalyzed oxidations. The physiologically irreversible sulfoxidation and covalent binding of quinones to the cysteines was not detectable. It is therefore expected that, in cells, oxidized *E. coli* thioredoxin will be reduced by NADPH/thioredoxin reductase back to sulfhydryls. Human thioredoxin, however, contains three structural cysteine residues, Cys61, -68, and -72, besides the catalytic cysteine pair. These cysteines are sensitive to redox modification, and their oxidation probably regulates the activity of the thioredoxin reductase/thioredoxin system (Ren et al., 1993). The reaction of the structural cysteines with phenoxyl radicals may be also important.

Since phenol is recycled, irreversible covalent modification of thioredoxin by phenol oxidation products (quinones) will occur only if the oxidation of phenols proceeds via another pathway (e.g., cytochrome P450-catalyzed oxygenation) without intermediate release of phenoxyl radicals. Isolation of thioredoxin from cells treated with phenolic compounds could very well be a means to determine whether the phenoxyl radical-dependent pathway of phenol activation is important, as has been suggested for myeloperoxidase-catalyzed reactions in bone marrow and inflammatory cells (Eastmond et al., 1986; Pueringer & Hunnionghake, 1992).

The reversibility of the radical-mediated oxidation of vicinal cysteines to disulfides can be contrasted with reactions of isolated cysteines. Radical-catalyzed oxidation of GSH

and other isolated sulfhydryls yields concentration-dependent amounts of sulfoxy derivatives, mainly sulfonic acid (Winterbourn & Metodiewa, 1994; Zhang et al, 1994). The radical-catalyzed oxidation of isolated protein sulfhydryls may also result in irreversible conversion of cysteine to sulfonic acid (Coan et al., 1992). The oxidation of vicinal cysteines will be therefore perceived as oxidative stress (decrease in concentrations of reductants, e.g., NADPH, without direct modification of macromolecules), while oxidation of single sulfhydryls may result in oxidative injury due to irreversible modification of activity of sensitive proteins. For example, we have observed the inhibition of cytochrome P-450 reductase in reaction with phenol/HRP/H_2O_2. The inhibition was not reversible by DTT and likely was a result of sulfoxy derivatization of cysteine 565 involved in NADPH binding (unpublished results). The reversibility of thioredoxin oxidation is an important result which shows that the radical-mediated oxidations are not necessarily destructive events but rather regulatory processes reflected in the activity of redox-sensitive enzymes, protein channels, and receptors.

REFERENCES

Asmus, K. D. (1990) *Methods Enzymol. 186*, 168−180.

Aust, S. D., Chignell, C. F., Bray, T. M., Kalyanaraman, B., & Mason, R. P. (1993) *Toxicol. Appl. Pharmacol. 120* (2), 168−178.

Baek, H. K., & Van Wart, H. E. (1992) *J. Am. Chem. Soc. 114*, 718−725.

Brabec, V., & Mornstein, V. (1980) *Biophys. Chem. 12*, 159−165.

Bray, T. M., & Taylor, C. G. (1993) *Can. J. Physiol. Pharmacol. 71* (9), 746−751.

Cadenas, E., & Sies, H. (1985) *Adv. Enzyme Regul. 23*, 217−237.

Chance, B., Sies, H., & Boveris, A. (1979) *Physiol. Rev. 59*, 527−605.

Claiborne, A., Ross, R. P., & Parsonage, D. (1992) *Trends Biochem. Sci. 17* (5), 183−186.

Coan, C., Ji-Ying, J., Hideg, K., & Mehlhorn, R. J. (1992) *Arch. Biochem. Biophys. 295* (2), 369−378.

DeFelippis, M. R., Murthy, C. P., Faraggi, M., & Klapper, M. H. (1989) *Biochemistry 28*, 4847−4853.

Derman, A. I., Prinz, W. A., Belin, D., & Beckwith, J. (1993) *Science 262*, 1744−1747.

Dyson, H. J., Tennant, L. L., & Holmgren, A. (1991) *Biochemistry 30*, 4262−4268.

Eastmond, D. A., Smith, M. T., Ruzo, L. O., & Ross, D. (1986) *Mol. Pharmacol. 30*, 674−679.

Eklund, H., Gleason, F. K., & Holmgren, A. (1991) *Proteins: Struct., Funct., Genet. 11*, 13−28.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.

Ericson, M. L., Horling, J., Wendel-Hansen, V., Holmgren, A., & Rosen, A. (1992) *Lymphokine Cytokine Res. 11* (5), 201−210.

Faulkner, K., & Fridovich, I. (1993) *Free Rad. Biol. Med. 15*, 447−451.

Foti, M., Ingold, K. U., & Lusztyk, J. (1994) *J. Am. Chem. Soc. 116*, 9440−9447.

Gilbert, H. F. (1990) *Adv. Enzymol. 63*, 69−172.

Holmgren, A. (1979) *J. Biol. Chem. 254* (18), 9113−9119.

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237−271.

Holmgren, A. (1989) *J. Biol. Chem. 264* (24), 13963−13966.

Holmgren, A., & Luthman, M. (1979) *Biochemistry 17*, 4071−4077.

Holmgren, A., Soderberg, B. O., Eklund, H., & Branden, C. I. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2305−2309.

Jeng, M. F., Campbell, A. P., Begley, T., Holmgren, A., Case, D. A., Wright, P. E., & Dyson, H. J. (1994) *Structure 2*, 853−858.

Kagan, V. E., Yalowich, J. C., Day, B. W., Goldman, R., Gantchev, T. G., & Stoyanovsky, D. A. (1994) *Biochemistry 33*, 9651−9660.

Kalyanaraman, B. (1982) in *Reviews in Biochemical Toxicology* (Hodgson, E., Bend, J. R., & Philpot, R. M., Eds.) pp 73−139, Elsevier, New York.

Katki, A. G., Kalyanaraman, B., & Sinha, B. K. (1987) *Chem.-Biol. Interact. 62*, 237−247.

Katti, S. K., LeMaster, D. M., & Eklund, H. (1990) *J. Mol. Biol. 212*, 167−184.

Kistner, A., Sanders, D., & Habermann, E. (1993) *Toxicon 31* (11), 1423−1434.

Kuriyan, J. (1993) *Curr. Sci. 64* (2), 85−95.

Lind, J., Shen, X., Eriksen, T. E., & Merenyi, G. (1990) *J. Am. Chem. Soc. 112*, 479−482.

Little, C., & O'Brien, P. J. (1969) *Eur. J. Biochem. 10*, 533−538.

Lozano, R. M., Yee, B. C., & Buchanan, B. B. (1994) *Arch. Biochem. Biophys.* 309(2), 356−362.

Mason, R. P., & Rao, D. N. R. (1990) *Methods Enzymol. 186*, 318−330.

Meister, A., & Anderson, M. E. (1983) *Annu. Rev. Biochem. 52*, 711−760.

Miranda-Vizuete, A., Martinez-Galisteo, E., Aslund, F., Lopez-Barea, J., Pueyo, C., & Holmgren, A. (1994) *J. Biol. Chem. 269* (24), 16631−16637.

Moore, E. C., Reichard, P., & Thelander, L. (1964) *J. Biol. Chem. 239*, 3445−3452.

Moore, K. L., Moronne, M. M., & Mehlhorn, R. J. (1992) *Arch. Biochem. Biophys. 299* (1), 47−56.

Niki, E. J., Tsuchiya, R., Tanimura, R., & Kamiya, Y. (1982) *Chem. Lett. 6*, 789−792.

Pacifici, R. E., Salo, D. C., & Davies, K. J. A. (1989) *Free Rad. Biol. Med. 7*, 521−536.

Pueringer, R. J., & Hunnionghake, G. W. (1992) *Am. J. Med. 92*, 32S-38S.

Rao, D. N. R., Fischer, V., & Mason, R. P. (1990) *J. Biol. Chem. 265* (2), 844−847.

Ren, X., Bjornstedt, M., Shen, B., Ericson, M. L., & Holmgren, A. (1993) *Biochemistry 32*, 9701−9708.

Romero, F. J., Ordonez, I., Arduini, A., & Cadenas, E. (1992) *J. Biol. Chem. 267* (3), 1680−1688.

Sabourin, P. J., Muggenburg, B. A., Couch, R. C., Lefler, D., Lucier, G., Birnbaum, L. S., & Henderson, R. F. (1992) *Toxicol. Appl. Pharmacol. 114*, 277−284.

Sakurada, J., Sekiguchi, R., Sato, K., & Hosoya, T. (1990) *Biochemistry 29*, 4093−4098.

Salamon, Z., Gleason, F. K., & Tollin, G. (1992) *Arch. Biochem. Biophys. 299* (1), 193−198.

Schreiber, J., Foureman, G. L., Hughes, M. F., Mason, R. P., & Eling, T. E. (1989) *J. Biol. Chem. 264* (14), 7936−7943.

Siedler, F., Rudolphbohner, S., Doi, M., Musiol, H. J., & Moroder, L. (1993) *Biochemistry 32*, 7488−7495.

Stadtman, E. R. (1993) *Annu. Rev. Biochem. 62*, 797−821.

Surdhar, P. S., & Armstrong, D. A. (1986) *J. Phys. Chem. 91*, 6532−6537.

Thelander, L., & Reichard, P. (1979) *Annu. Rev. Biochem. 48*, 133−135.

Tyurina, Y. Y., Tyurin, V. A., Yalowich, J. C., & Kagan, V. E. (1994) *Toxicol. Appl. Pharmacol.* (in press).

Wayner, D. D., Burton, G. W., Ingold, K. U., Barclay, L. R., & Locke, S. J. (1987) *Biochim. Biophys. Acta 924* (3), 408−419.

Williams, C. H., Jr. (1976) *Enzymes 13*, 89−173.

Winger, B. E., Hofstadler, S. A., Bruce, J. E., Udseth, H. R., & Smith, R. D. (1993) *J. Am. Soc. Mass. Spectrom. 4*, 566−577.

Winterbourn, C. C., & Metodiewa, D. (1994) *Arch. Biochem. Biophys. 314* (2), 284−290.

Yim, M. B., Chae, H. Z., Rhee, S. G., Chock, P. B., & Stadtman, E. R. (1994) *J. Biol. Chem. 269* (3), 1621−1626.

Zhang, X., Zhang, N., Schuchmann, H.-P., & von Sonntag, C. (1991) *J. Phys. Chem. 95*, 4718−4722.

Zhang, X., Zhang, N., Schuchmann, H.-P., & von Sonntag, C. (1994) *J. Phys. Chem. 98*, 6541−6547.

BI942631+