0026-895X/87/040417-05$00.00/0

MOLECULAR PHARMACOLOGY, 31:417–421

Oxidation of Thiol Drugs and Biochemicals by the Lactoperoxidase/Hydrogen Peroxide System

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Received March 21, 1986; Accepted January 5, 1987

SUMMARY

The thiol moiety is prone to oxidative free radical formation, which may be important in mediating the toxicity of some thiol-containing compounds. The oxidation of the compounds cysteine, cysteamine, *N*-acetylcysteine, glutathione, penicillamine, and captopril were studied using ESR and oxygen uptake techniques. Lactoperoxidase, with hydrogen peroxide to provide oxidizing equivalents, was used to initiate the oxidation. The reaction appears to be strongly peroxide dependent, with either exogenous H_2O_2 or thiol-derived peroxide driving the reaction.

Thiol-containing compounds are important in many biochemical and pharmacological reactions: the structural role of disulfide bonds in proteins, the effects and side effects of thiol-containing drugs, and the protective effect of reduced cellular glutathione. In general, the reductive activity of thiols is considered beneficial: cysteine and cysteamine are radioprotectants (1, 2), cysteamine and *N*-acetylcysteine are used as antidotes in acetaminophen overdose (3, 4), and glutathione is a major cellular defense against reactive species in the body. Yet the ability to reduce reactive species implies a susceptibility to oxidation, which may produce potentially toxic sulfur-centered free radicals and other active species (5). We have therefore studied a series of thiol compounds of pharmacological significance to determine pathways of oxidation.

Although many thiol oxidation studies have examined the mechanisms of radiation-initiated (6–8) or metal ion-catalyzed (9–15) events, these conditions are hardly physiological. We have chosen to use lactoperoxidase to initiate the reaction, with hydrogen peroxide providing the oxidizing equivalents. This peroxidase is widely distributed in milk, saliva, and tears (16–18), and has an optical spectrum with a Soret band similar to that of thyroid peroxidase, intestinal peroxidase, uterine peroxidase, and eosinophil peroxidase (19). As such, lactoperoxidase appears to be a useful prototype for most mammalian hemoprotein peroxidases. The thiols which we used were cysteine, cysteamine, penicillamine, *N*-acetylcysteine, glutathione, and captopril.

K. T. was supported by National Institutes of Health Grant 5 T32 ES 07126.
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Despite the protective roles mentioned above (1–3), both cysteine and cysteamine produce toxicity in cell culture via a free radical mechanism (11, 12, 14). Cysteine also produces a positive result in the Ames assay for mutagenicity (20). Caution is thus dictated in the use of these compounds as protectants either *in vitro* or *in vivo*.

The cysteine analog penicillamine is an anti-inflammatory agent used in the treatment of arthritis (21) and a copper chelator used in the treatment of copper overdose, Wilson's disease, and schizophrenia (22, 23). The latter effect has been suggested as a radical-mediated reduction and chelation of nonspecifically bound copper (22). The antiarthritic action may be due to the inhibition of lymphocyte mitogenesis via H_2O_2 production, which has been demonstrated in copper-catalyzed penicillamine oxidation (15). It has also been suggested that penicillamine may potentiate acetaminophen nephrotoxicity (3). Skin rash is the most common side effect of this drug; however, blood dyscrasias, immune complex-mediated proteinuria, acute polyarthritis, and autoimmune syndromes such as lupus, Goodpasture's syndrome, polymycites, and myasthenia gravis appear to be the most serious (21). Each of these could conceivably be due to radical-mediated damage interfering with correct tissue recognition (24). Penicillamine also produces hypoglycemia in insulin-dependent diabetics (25), which may be related to penicillamine-induced autoantibodies to insulin (26).

To our knowledge, few major side effects of the acetaminophen antidote *N*-acetyl-cysteine have been reported, and none attributable to thiol activation.

Glutathione, as an endogenous cellular protectant, would be expected to show little toxicity at all, either *in vivo* or *in vitro*, yet it does test positive in the Ames assay (20).

ABBREVIATIONS: DTPA, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide.

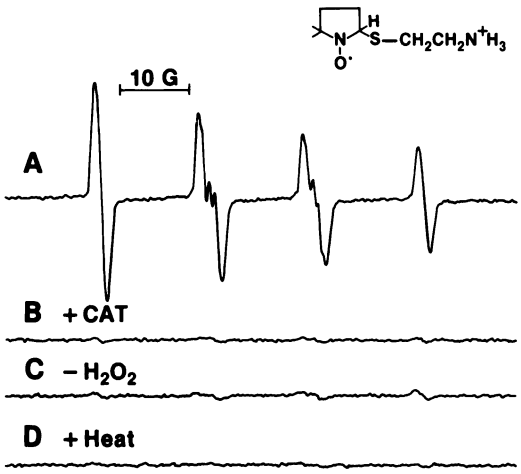

Fig. 1. ESR spectra of DMPO-*SR* adduct of cysteamine. A. Ten mM cysteamine, 0.1 mg/ml lactoperoxidase, 100 μM hydrogen peroxide, and 135 mM DMPO in phosphate buffer (1 mM DTPA), pH 7.4. B. Same as A, but with 5000 units/ml catalase (*CAT*). C. Same as A, but without hydrogen peroxide. D. Same as A, but using heat-denatured enzyme. Instrumental conditions: microwave power 20 mW; modulation amplitude 0.16 G; time constant 0.2 sec; scan rate 60 G/min; gain 1.25×10^5.

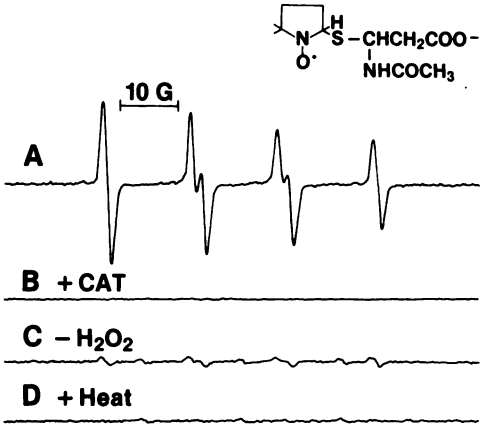

Fig. 2. ESR spectra of DMPO-*SR* adduct of *N*-acetylcysteine. A. Ten mM *N*-acetylcysteine, 0.1 mg/ml lactoperoxidase, 200 μM hydrogen peroxide, and 135 mM DMPO in phosphate buffer (1 mM DTPA), pH 7.4. B. Same as A, but with 5000 units/ml catalase (*CAT*). C. Same as A, but without hydrogen peroxide. D. Same as A, but using heat-denatured enzyme. Instrumental conditions: microwave power 20 mW; modulation amplitude 0.66 G; time constant 0.128 sec; scan rate 50 G/min; gain 2.5×10^3.

Captopril, an angiotensin-converting enzyme inhibitor, is used as a hypertensive agent. Side effects include rash, granulocytopenia, neutropenia, and bone marrow aplasia. The hemotologic effects are rare and generally accompany renal insufficiency, collagen-vascular disease, complex drug regimens, or other complicating factors (27–32). Captopril, like penicillamine (25), produces possibly immune-related hypoglycemia in insulin-dependent diabetics (33), although Benson *et al.* (26) found no insulin antibodies during captopril treatment. Kallenberg (34) associated some of the side effects of captopril with antinuclear antibodies found during captopril treatment. As discussed for penicillamine, immune effects may be free radical related. The sulfur atom helps to position the molecule in the enzyme's active site by ligating to a protein-bound zinc ion (35). No radical formation is necessary for this pharmacological effect, but captopril is reported to be chemically unstable in biological fluids, forming a dimer (36). This product could easily

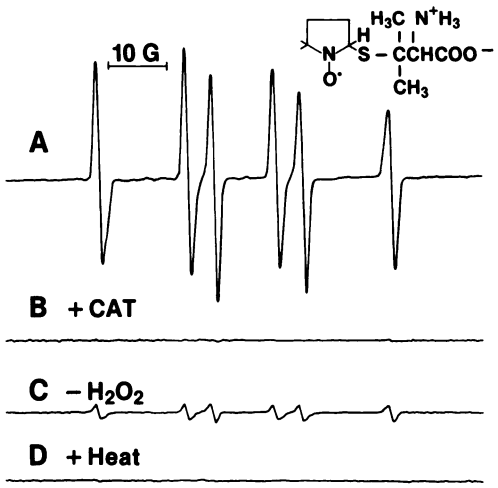

Fig. 3. ESR spectra of DMPO-*SR* adduct of penicillamine. A. Ten mM penicillamine, 0.1 mg/ml lactoperoxidase, 100 μM hydrogen peroxide, and 135 mM DMPO in phosphate buffer (1 mM DTPA), pH 7.4. B. Same as A, but with 5000 units/ml catalase (*CAT*). C. Same as A, but without hydrogen peroxide. D. Same as A, but using heat-denatured enzyme. Instrumental conditions: microwave power 20 mW; modulation amplitude 0.66 G; time constant 0.128 sec; scan rate 50 G/min; gain 2.0×10^3.

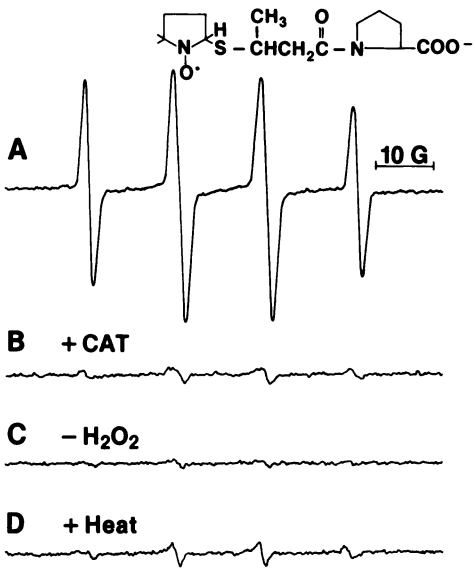

Fig. 4. ESR spectrum of DMPO-*SR* adduct of captopril. A. Ten mM captopril, 0.1 mg/ml lactoperoxidase, 200 μM hydrogen peroxide, and 135 mM DMPO in phosphate buffer (1 mM DTPA), pH 7.4. B. Same as A, but with 5000 units/ml catalase (*CAT*). C. Same as A, but without hydrogen peroxide. D. Same as A, but using heat-denatured enzyme. Instrumental conditions: microwave power 20 mW; modulation amplitude 0.40 G; time constant 0.5 sec; scan rate 30 G/min; gain 2.5×10^5.

result from thiyl free radical formation followed by dimerization.

Materials and Methods

L-Cysteine, cysteamine, penicillamine, *N*-acetylcysteine, reduced glutathione, DTPA, catalase, lactoperoxidase, and DMPO were all obtained from Sigma Chemical Co. (St. Louis, MO). The DMPO was purified by vacuum distillation and stored under nitrogen at −20°. Hydrogen peroxide (30%, ACS certified) was obtained from Fisher Scientific Co. (Pittsburgh, PA). Superoxide dismutase was from Diagnostic Data Inc. (Mountain View, CA). Captopril was a generous gift from E. R. Squibb and Sons (Princeton, NJ).

TABLE 1
Hyperfine splitting constants of thiyl adducts of DMPO[a]

Thiol	a^N	a^H_β	a^H	Linewidth used in simulation
				G
Cysteamine	15.2	17.0	0.72_5[b]	0.8
N-Acetylcysteine	15.1 (15.0)[c]	16.7 (16.8)[c]	0.48[b]	0.9
Penicillamine	15.3_5	20.0		1.2
Captopril	15.4	16.0		1.2
Cysteine	15.1 (15.6, 15.2)[d] (15.3)[e] (15.3)[f] (15.2)[c]	17.4 (17.7, 16.7)[d] (17.0)[e] (17.2)[f] (17.0)[c]	0.60[b]	0.8
Glutathione	15.1 (15.0)[c] (15.4)[g]	16.0 (16.3)[c] (16.2)[g]	0.64[b]	0.7

[a] Values were obtained by computer simulation (in gauss) in buffer or water.
[b] Two protons; γ-H on DMPO ring or δ-H on alkylthiyl group.
[c] Ref. 41.
[d] Ref. 39.
[e] Ref. 14.
[f] Ref. 38.
[g] Ref. 37.

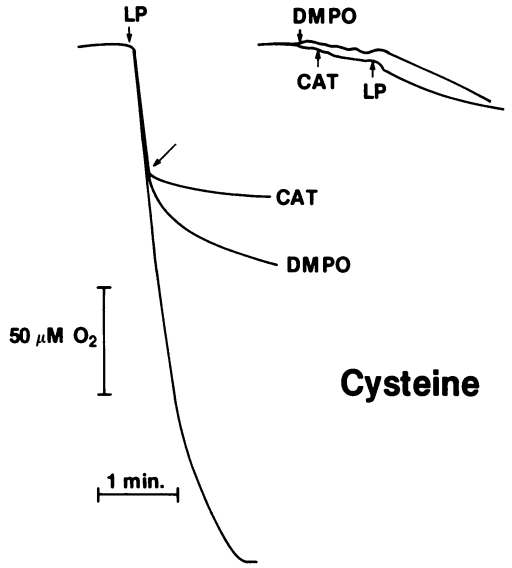

Fig. 5. Oxygen uptake measurements with cysteine. All incubations contain 10 mM cysteine in phosphate buffer (1 mM DTPA), pH 7.4. Lactoperoxidase (*LP*, 0.1 mg/ml), catalase (*CAT*, 5000 units/ml), and DMPO (135 mM) were added at the appropriately labeled *arrows*.

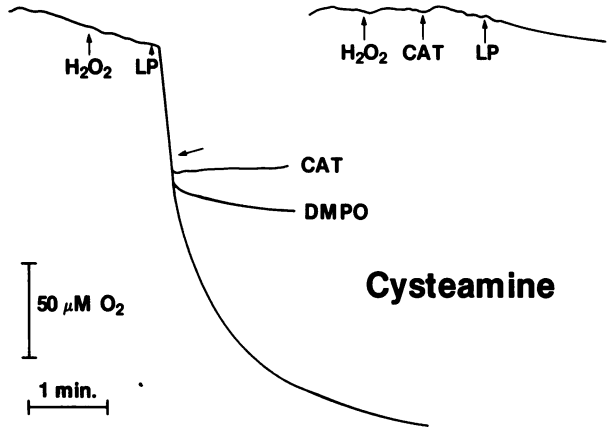

Fig. 6. Oxygen uptake measurements with cysteamine. All incubations contain 10 mM cysteamine in phosphate buffer (1 mM DTPA), pH 7.4. Hydrogen peroxide (100 μM), lactoperoxidase (*LP*, 0.1 mg/ml), catalase (*CAT*, 5000 units/ml), and DMPO (135 mM) were added at the appropriately labeled *arrows*.

ESR measurements were made at room temperature on either an IBM ER-200D or a Varian E-109 spectrometer. Samples were aspirated into an aqueous flat cell in place in a TM_{110} cavity. Splitting constants were determined with computer simulation using a Nicolet 1180 computer. Oxygen uptake studies were done at 25° with a YSI model 53 oxygen monitor.

The following concentrations were used: 0.1 M sodium phosphate buffer (pH 7.4) with 1 mM DTPA, 10 mM substrate, 0.1 mg/ml lactoperoxidase, 100 or 200 μM H_2O_2, 5000 units/ml catalase, and 135 mM DMPO where appropriate. For enzyme controls, lactoperoxidase was denatured in a capped vial in boiling water for 5 min. All buffers were prepared with 1 mM DTPA to keep metal-catalyzed autoxidation to a minimum.

Results

The ESR spectra of the DMPO adducts formed by the oxidation of cysteamine, *N*-acetylcysteine, penicillamine, and captopril by lactoperoxidase are shown in Figs. 1–4. The ESR spectra for the glutathione and cysteine thiyl adducts were the same as those previously reported (14, 37, 38). Hyperfine splitting constants for the thiyl adducts formed in this study are given in Table 1 along with values previously reported in the literature (shown in parentheses). The gamma hydrogen splittings are smaller than the linewidth and, except for cysteamine, affect peak shape rather than peak position. The penicillamine spectrum (Fig. 3) appears to be the result of two radicals, the DMPO-thiyl adduct from penicillamine and the DMPO-OH adduct with splitting constants of $a^N = a^H_\beta = 14.9$ G (14). The addition of the DMPO-OH adduct spectrum accounts for the asymmetric appearance of the lines.

As can be seen from Figs. 1–4, catalase completely inhibited radical formation from cysteamine, *N*-acetylcysteine, and penicillamine, and significantly diminished it from captopril. The glutathione-derived radical was greatly decreased, and the cysteine-derived radical signal was totally absent in the presence of catalase (data not shown). Cysteine and penicillamine produced ESR spectra in the absence of exogenous hydrogen peroxide (Fig. 3C). Heat-denatured enzyme was inactive, indicating that hydrogen peroxide alone does not effect the oxidation, i.e., the oxidation is enzymatic.

The rates of oxygen uptake differ from compound to compound. Captopril, glutathione, and *N*-acetylcysteine show essentially no oxygen uptake in the presence of 100 μM hydrogen peroxide (data not shown). Cysteine and penicillamine both followed the same pattern as illustrated in Fig. 5 (penicillamine

data not shown), showing rapid oxygen uptake without the addition of exogenous hydrogen peroxide. In the case of cysteamine (Fig. 6), hydrogen peroxide greatly increased the oxygen consumption rate. Catalase and DMPO effectively prevented oxygen uptake by all three thiols if added before the lactoperoxidase, and stopped oxygen uptake if added during the course of the reaction. Heat-denatured lactoperoxidase did not catalyze any oxygen uptake.

Discussion

ESR and oxygen uptake studies confirm that lactoperoxidase/hydrogen peroxide/thiol incubations produce free radicals which react with oxygen or DMPO. The DMPO-cysteine thiyl radical adduct has been unambiguously assigned by mass spectroscopy and nuclear magnetic resonance spectroscopy of the corresponding hydroxylamine (14). The hyperfine coupling constants for the DMPO-glutathione thiyl radical adduct are also known (37, 39). Splitting constants of the other three DMPO-thiyl adducts in this paper are similar.

The effect of catalase on each of the thiol incubations shows that the lactoperoxidase (LP)-catalyzed formation of $RS\cdot$ is hydrogen peroxide dependent. However, both penicillamine (Fig. 3) and cysteine produce ESR spectra and rapid oxygen uptake (Fig. 5) without exogenous hydrogen peroxide. Presumably, penicillamine and cysteine autooxidize to form significant hydrogen peroxide to drive their own initial oxidations.

$$RSH \xrightarrow[H_2O_2]{LP} RS\cdot \quad (1)$$

$$RS\cdot + RS^- \rightarrow R\dot{S}\bar{S}R \quad (2)$$

$$R\dot{S}\bar{S}R + O_2 \rightarrow RSSR + \dot{O}_2^- \quad (3)$$

$$2\dot{O}_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

Although cysteamine oxidation requires exogenous hydrogen peroxide (Figs. 1 and 6), the rapid oxygen uptake in this system suggests that, even though initiation may require an external source of oxidizing equivalents, maintenance of the reaction via hydrogen peroxide production does occur. *N*-Acetylcysteine, glutathione, and captopril, which consume little oxygen under the conditions of this study, should be dependent upon exogenous hydrogen peroxide. This distinction would become significant under normal *in vivo* conditions where, except in areas of inflammation, high concentrations of hydrogen peroxide are not likely to be found (40). A self-sustaining cascade reaction which produces and consumes highly reactive oxygen metabolites is consistent with cysteine, penicillamine, and cysteamine data shown here and could be of toxicological importance.

In short, the lactoperoxidase-catalyzed *in vitro* oxidation of a series of thiol compounds results in thiol radical formation and, in some cases, reduction of oxygen to hydrogen peroxide. In particular, cysteine, penicillamine, and cysteamine could be oxidized by this pathway under physiological conditions, resulting in self-sustaining free radical chain reactions.

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