An Alkyl Hydroperoxide Reductase from Salmonella typhimurium
Involved in the Defense of DNA against Oxidative Damage

PURIFICATION AND PROPERTIES*

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A peroxide reductase (peroxidase) which converts lipid hydroperoxides and other alkyl hydroperoxides to the corresponding alcohols, using either NADH or NADPH as the reducing agent, has been identified in both Salmonella typhimurium and Escherichia coli. This enzyme is shown to play a role in protecting against alkyl hydroperoxide mutagenesis. To our knowledge this work represents the first description of an NAD(P)H peroxidase in enteric bacteria and the first reported bacterial peroxidase to exhibit high activity toward alkyl hydroperoxides. A high performance liquid chromatography-based assay for the alkyl hydroperoxide reductase has been developed by monitoring the reduction of cumene hydroperoxide, a model alkyl hydroperoxide. By using this assay, the enzyme has been purified from a S. typhimurium regulatory mutant, oxyR1, which overexpresses a number of proteins involved in defenses against oxidative damage, and which contains 20-fold more of the alkyl hydroperoxide reductase than the wild-type strain. The purified activity requires the presence of two separable components having subunit molecular weights of 22,000 and 57,000. The 57-kDa protein contains a bound FAD cofactor and can use either NADH or NADPH as an electron donor for the direct reduction of redox dyes, or of alkyl hydroperoxides when combined with the 22-kDa protein. This enzyme may thus serve as a prokaryotic equivalent to the glutathione reductase/glutathione peroxidase system in eukaryotes.

Incomplete reduction of molecular oxygen during respiration can lead to the generation of potent oxidizing agents (1). Subsequent oxidation of biological molecules can produce a variety of toxic by-products depending on the type of oxidant and on the site of attack. These products include epoxides, aldehydes, and alkyl hydroperoxides as well as a variety of nitrogen and sulfur oxidation products. In order to minimize the deleterious effects of such by-products, cells can destroy the oxidant either enzymatically (using enzymes such as superoxide dismutase or catalase) or non-enzymatically (using anti-oxidants such as β-carotene, glutathione, α-tocopherol, ascorbate, bilirubin, or urate). Should these first-line defenses fail the resulting damage can be repaired by a wide range of enzymes such as DNA repair enzymes, glutathione peroxidase, methionine sulfoxide reductase (1–3). In enteric bacteria, some of these defenses against oxidative stress are regulated in a coordinate fashion (4).

Treatment of Salmonella typhimurium with hydrogen peroxide induces a characteristic set of 30 proteins, nine of which are under the control of a positive regulatory gene, oxyR (4, 5). The proteins of this set which have been identified are involved in protection against oxidative damage. Identification of these proteins has been facilitated by isolation of a mutant strain of S. typhimurium, oxyR1 (4), which is resistant to killing and mutation by hydrogen peroxide and a number of other oxidizing agents including a wide range of alkyl hydroperoxides. The oxyR1 mutation causes the overproduction of at least nine proteins in S. typhimurium (4). Our early experiments demonstrated the presence of high levels of catalase in cell extracts of oxyR1, potentially explaining the resistance to hydrogen peroxide mediated killing and mutagenicity. Catalase, which efficiently decomposes hydrogen peroxide, can utilize methyl and ethyl hydroperoxides only in a slow peroxidatic reaction requiring an exogenous reductant such as ethanol or a redox dye. Bulkier hydroperoxides do not serve as substrates, however (6). This suggested that alkyl hydroperoxide resistance derives either from another protein capable of detoxifying these compounds, or overexpression of an activity which repairs some lethal and mutagenic damage. In order to understand the molecular basis of bacterial resistance to alkyl hydroperoxides, we isolated an activity responsible for detoxifying these compounds from extracts of the oxyR1 mutant. This paper describes the purification and initial characterization of a novel alkyl hydroperoxide (AHP)1 reductase.

EXPERIMENTAL PROCEDURES

Enzyme Assays—Alkyl hydroperoxide reductase was assayed in whole cells as described previously (4). Activity in crude cell extracts and during purification was assayed by the following modification of that method. A fresh stock solution of 0.05% cumene hydroperoxide (CHP) was prepared daily in 50 mM KPi buffer, pH 7.0, containing 15 mg/ml BSA. A second stock solution containing 10 mM MgCl2, 2

The abbreviations used are: AHP, alkyl hydroperoxide; CHP, cumene hydroperoxide; CA, cumyl alcohol (2-phenyl-2-propanol); DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); VBC, Vogel-Bonner citrate (8); BSA, bovine serum albumin; HPLC, high performance liquid chromatography.
mm NADP, 40 mM glucose-6-phosphate, and 2 units/ml Torula yeast glucose-6-phosphate dehydrogenase (Sigma, type XII) was prepared, and reactions were initiated by addition of 0.25 ml of each of the two stock solutions to a 3-ml conical test tube containing 5-50 μl of the sample to be assayed. Following a 20-min incubation at 37 °C, reactions were terminated by addition of 0.5 ml of HPLC-grade ethyl acetate to some of the samples. Following vortex mixing and centrifugation, reverse-phase HPLC analysis of the extracts for conversion of CHP to cumyl alcohol (CA) was performed as described below. Assays requiring complementation by affinity column pools contained either 0.3 mg of the blue agarose-nonbinding fraction (during purification of the F52a protein) or 0.15 mg of the blue agarose-binding fraction (during purification of the C22 protein).

The quantities of CA and CHP were determined using an isocratic HPLC system consisting of a Supelco 5-μm LC-18 (0.46 × 25-cm) octadecylsilicic column and a mobile phase containing 35.65% of acetonitrile/5 mM KPi, pH 7.0, at 2.0 ml/min. Only 10 μl of the ethyl acetate extract could be injected/assay as increasing sample volume markedly decreased resolution. Each analysis took 6-7 min. Commercial HCP contained a number of impurities as detected by HPLC analysis. The identities of the contaminants were determined by the coeluion of authentic standards.5 In order of elution after the ethyl acetate peak, the contaminants were: (CA), acetonaphone, and finally CA. Cymyl alcohol was present at about 5% the level of the hydroperoxide and this was subtracted when calculating the conversion of CHP to the CA.

Subsequent purification of the two components followed identical protocols. After ammonium sulfate concentration and dialysis, the blue agarose column nonbinding fraction was loaded onto a 17 cm (X 2.6-cm DEAE-cellulose column (DE52) and washed with 25 ml of 25 mM KPi buffer, pH 7.0. Enzyme activity was eluted with a 400-mU linear gradient from 25 to 200 mM KPi, pH 7.0. As these fractions had little or no reductase activity on their own, assays required the addition of the stock-binding fraction to locate the peak of activity. Fractions were pooled and then passed through a column of adenine-5’-monophosphate-agarose linked through the N(6) position with a six carbon spacer (5’-AMP-agarose, Sigma) to remove traces of the binding fraction component which tends to copurify with the nonbinding component. The activity, which did not bind to this affinity matrix, was pooled and stored frozen at −20 °C in aliquots.

Purification of the blue-dye agarose-binding fraction proceeded by identical means except that at the final step the protein mixture was loaded onto the 5’-AMP agarose column. Following thorough washing with 25 mM KPi, pH 7.0, the active component was eluted with 0.1 M NaB03, pH 9.0, containing 0.5 M NaCl. This protein was also stored frozen at −20 °C.

Protein concentrations were determined with a commercial reagent (Bio-Rad) that employs the method of Bradford (9). BSA was used as the standard.

Assays of Substrates Other Than Cumene Hydroperoxide—The reduction of alkyl hydroperoxides other than CHP was assayed by measuring the disappearance of K1-reaction product using a slight modification of the method of Tagaki et al. (10). Incubations (at 37 °C) contained the NADPH generating system, BSA, the KPi buffer, and the peroxide of interest at 2 mM. Reactions were initiated by addition of enzyme, either the crude 35-55% ammonium sulfate fraction or a mixture of the purified components. Prior to enzyme addition, and at 5, 10, and 20 min after addition, 30-μl aliquots were removed from each incubation and added to 1.5 ml of a 2:1 mixture of glacial acetic acid/chloroform. The peroxide content of these samples was assayed by the addition of 0.25 ml of 60% w/v sodium dodecyl sulfate (SDS) to 0.8 ml of KPi buffer. 150 μl of this mixture was added to 150 μl of deionized water in the presence of 10 μl of 0.2% 2,2’-diamino-4-amino-6-(hydroxy-1-naphthylazo)benzene (Sigma, 2 mm). The tubes were mixed and degassed with 20 μl of argon before addition of 0.1 ml of a 0.1% aqueous solution of sodium acetate was added and mixed with argon bubbling. The samples were briefly centrifuged to separate the layers and the 358 nm absorbance of the upper layer was determined.

DTNB Titrations—Determination of the content of free sulfhydryls was carried out using the method of Habeeb (11) modified to conserve protein. 2-5 nmol of each of the protein subunits was mixed with 250 μl of the sodium dodecyl sulfate/NADP/NADPH (5′,5′-dithiobis(2-nitrobenzoic acid)) reagent, and the absorbance at 412 nm was compared with that of a no-protein blank. For experiments where chemical reduction preceded the DTNB reaction, the protein was incubated with the reductant for 1 h at room temperature and then precipitated at 0 °C with 5% trichloroacetic acid. Following three washes with 5% trichloroacetic acid to remove the reducing agent, the pellet was dissolved in 1.2 ml of the reaction mixture and the absorbance at 412 nm was measured. Thiol concentration was calculated from a standard curve using β-mercaptoethanol and this method gave the same values as the use of the extinction coefficient (11).

Materials—13-Hydroxyperoxynitric acid was enzymatically synthesized from nitric acid using soybean lipoxidase (Sigma) (12). 5-Hydroperoxyethyl uracil was synthesized from the corresponding alcohol (13) cis-5,6-Dihydro-6-hydroperoxy-5-hydroxythymine was synthesized from cis-thymine glycol (cis-TG) using a similar method (14). Benzylhydroperoxide was prepared from the corresponding mesylate (15). p-Menthane hydroperoxide (55% in p-methanone) and ethyl hydroperoxide (10% aqueous) were obtained from Polysciences, Inc. (Warrington, PA). Cumene hydroperoxide was purchased from Pfaltz & Bauer (Whitehouse Station, NJ). Hydrogen peroxide (30%) and t-butyl hydroperoxide (70% aqueous) were purchased from Sigma.

Purified proteins used in the reconstitution experiments were derived from either Miles Laboratories (for the BSA) or from Sigma.

RESULTS

Mutagenticity of Peroxides in Strains Carrying the oxyR1 Allele—S. typhimurium strains carrying the hisG428 mutation

Bacterial Alkyl Hydroperoxide Reductase
are susceptible to reversion by a wide range of oxidizing agents including hydrogen peroxide, t-butyl hydroperoxide, and CHP (16, 17). In order to assess the effect of the oxyR1 allele on mutagenesis induced by CHP and t-butyl hydroperoxide, the reversion frequency for a strain (TA4118) containing hisG428, oxyR1, and the pKM101 plasmid was compared to the reversion frequency for an otherwise isogenic strain carrying the wild type oxyR* allele (TA4117). As shown in Fig. 1, the induced mutagenicity of the two alkyl hydroperoxides is diminished about 10-fold by the presence of oxyR1. The mutagenicity of low concentrations of hydrogen peroxide was also reduced 4–5-fold in TA4118 when compared to the control (data not shown).

Resistance to alkyl hydroperoxide mutagenicity could derive either from detoxification of the mutagen or repair of the resultant DNA damage. In order to assess the former possibility, whole cells were analyzed for their ability to decompose CHP.

Whole Cells and Cell-free Extracts of S. typhimurium Catalyze Reduction of Cumene Hydroperoxide—Cumene hydroperoxide was chosen as the model substrate for the following reasons. Past work with tester strains TA102 and TA104 indicated that it was an effective mutagen and was therefore likely to enter the cell (16, 17). Limited solubility of the CHP and corresponding CA in aqueous solution favors their partitioning into an organic phase such as ethyl acetate, facilitating HPLC analysis by separating them from proteins, cell debris, and water-soluble compounds which might otherwise interfere with the assay. Finally, the phenyl ring provides a UV chromophore allowing sensitive quantitation of substrate and product.

**Fig. 1. Peroxide-induced mutagenesis in oxyR1 and LT2 strain backgrounds.** Tester strains TA4117 (○) and TA4118 (■) were constructed by P22-mediated transduction of the hisG428 mutation, linked to a Tn10, into S. typhimurium strains LT2 (yielding TA4117) or oxyR1 (yielding TA4118). The strains also contain the pKM101 plasmid carrying the genes necessary for error-prone DNA repair. Reversion assays were performed in triplicate as described by Levin et al. (13, 14), using the indicated amounts of either CHP (panel A) or t-BHP (panel B). Total revertants/plate are plotted in all cases. For TA4117 the spontaneous reversion frequency is 25/plate, for TA4118 it is 16/plate.

In the presence of glucose, whole cells of oxyR1 demonstrated a 10-fold higher rate of CHP reduction than wild-type cells. Incubation of washed whole cells with CHP suspended in VBC salts resulted in little time-dependent reduction to the alcohol unless an energy source such as 0.4% glucose was added (Fig. 2). This stimulation by glucose suggested a need for an energy source which would lead to intracellular production of reducing equivalents such as NADPH or NADH.

To test this hypothesis, a cell-free extract of oxyR1 was prepared and assayed for its ability to catalyze the reduction of CHP. In the presence of either 1.5 mM NADH or NADPH and 1.6 mM CHP, a 2 mg/ml solution of oxyR1 extract reduced the 0.4 mM (using NADH) or 0.5 mM (using NADPH) of the peroxide within 30 min. Under identical conditions, extracts of wild-type S. typhimurium exhibited little reduction.

The Reduction of Cumene Hydroperoxide Is Due to an Enzyme That Is Distinct from Bacterial Catalase—Incubation of CHP and oxyR1 cell extract in the presence of tetramethylphenylene diamine, a chromogenic reducing agent used in assays of heme-dependent peroxidases (18), caused no reduction in excess of the control. This suggests that the reduction of alkyl hydroperoxides is due to a NAD(P)H-dependent enzyme that is distinct from bacterial catalase (which also possesses a peroxidase activity (19, 20)).

To further test the hypothesis that AHP reductase was distinct from catalase, oxyR1 crude extracts were fractionated with ammonium sulfate prior to an assay for NADPH-dependent hydroperoxide reduction. Overproduction of catalase in oxyR1 by 30–50-fold results in its precipitation in the 0–25% ammonium sulfate fraction rather than in the 25–50% fraction used for its precipitation in wild-type bacteria (19, 20). The low salt fraction, which contained 95% of the catalase activity, had only 5–10% of the reductase activity while the 25–50% fraction, with only 5% of the catalase activity, had the majority of the CHP-reducing activity (data not shown). Final confirmation that this peroxide reduction is distinct...
from catalase came from assays of oxyR1 cells containing a deletion of the katG locus (coding for the oxyR1-induced catalase). oxyR1 ΔkatG exhibits the wild-type level of catalase but the same level of CHP reduction as the parent oxyR1 (data not shown).

Purification of the NAD(P)H-dependent Alkyl Hydroperoxide Reductase—Initial attempts to purify the enzyme from oxyR1 extracts were unsuccessful due to progressive loss of activity during chromatography. This was demonstrated to be due to a requirement for two separable components. When a number of affinity resins were screened for their ability to bind the reductase, two (Affi-Gel Blue and 5'-AMP agarose) catalase). oxyR1 AkatG exhibits the wild-type level of catalase deletion of the katG locus (coding for the oxyR1-induced proteins). Fifty g of

Table I and Fig. 3 show a representative purification of the two components. The details of the purification are given under “Experimental Procedures.” The activity is taken as 100% after the first affinity column since this is the stage at which reconstitution assays are begun. From this stage to the final protein, the recovery of activity was about 50% for both proteins. Fifty g of S. typhimurium oxyR1 cell paste yielded 100 mg of highly purified nonbound component and 8.5 mg of the bound component.

Fig. 3 shows that the purified proteins have subunit molecular weights of 22,000 for the nonbound component and 57,000 for the bound component. The final samples are >95% pure as judged by densitometer scans even though these overloaded gels showed several minor contaminants.

Identification of the Alkyl Hydroperoxide Reductase Components as the oxyR-regulated Proteins C22 and F52a—Exposure of S. typhimurium to 60 μM hydrogen peroxide results in the oxyR-dependent induction of the nine proteins that are overproduced constitutively in the oxyR1 mutant (4). In order to determine which of these proteins correspond to the purified proteins, two-dimensional gel electrophoresis (22) of whole cell extracts spiked with each component was carried out. As seen in Fig. 4A, L/T2 spiked with the 57-kDa (affinity resin bound) protein shows a spot coincident with the oxyR1 species “F52a” and the Affi-Gel nonbound, 22-kDa protein comigrates with protein “C22” (Fig. 4B). Both of these proteins are induced by low levels of hydrogen peroxide or CHP, and F52a (but not C22) is heat-inducible in an oxyR-dependent fashion (5).

Reconstitution of Alkyl Hydroperoxide Reductase Activity with Purified Components—During purification of each component, the complementary activity in the assay was provided by crude fractions derived from the first affinity chromatography step. In order to determine if the two purified proteins were sufficient for the activity, these were combined and assayed. Combination of the two components alone gave little activity. However, the addition of 10 mg/ml of BSA (Miles Laboratories; Pentex fraction, crystallized) gave approximately a 10-fold stimulation in the rate of CHP reduction. Neither BSA alone, nor BSA with either individual component, produced any activity. Titration of the assay with increasing concentrations of BSA indicated that activity increases to a plateau at about 2–3 mg/ml of added protein. To determine if the stimulatory effect of BSA is due to either a protective effect contributed by protein thiols or to the addition of albumin-bound lipids, both thiol-modified BSA and fatty-acid-free BSA were tested. Both types of BSA stimulated the assay to the same extent as the crystalline product (data not shown). A variety of other proteins and reagents were assayed for their effects on activity (Table II). No other protein worked as well as BSA; however, some stimulation was observed with conalbumin, ovalbumin, and thyroglobulin. No stimulation was seen with unilamellar or multilamellar liposomes of dimyristoyl phosphatidylcholine (25, 26), or with the detergent Triton X-100. Octyl-glucopyranoside, glycerol, apo-ferritin, and α-amylose appeared to be somewhat inhibitory. Substitution of pH 7.0 KPi with a variety of other buffers in the pH range of 5.5–9.0 had no effect (data not shown).

Three reagents commonly used for inhibiting sulfhydryl-dependent enzyme reactions were also assayed for their effects.

| Table I | Summary of purifications of alkyl hydroperoxide reductase components |
|---------|---------------------------------------------------------------------|
| Step    | Total protein | Specific activity | Relative yield |
| Blue agarose-retained | mg | μmol CA/mg/30 min | % |
| Initial blue agarose | 189 | 14 | 100 |
| DEAE pool | 16.5 | 92 | 58 |
| Bio-Gel A0.5m pool | 9.1 | 130 | 45 |
| Final protein | 8.5 | 140 | 46 |
| Blue agarose non-binding | 448 | 9.7 | 100 |
| Initial blue agarose wash-through | 186 | 12.0 | 51 |
| DEAE pool | 105 | 21.3 | 52 |
| Bio-Gel A0.5m pool | 100 | 20.0 | 46 |

* All assays of the blue agarose retentate fractions were performed in the presence of 0.3 mg of the pooled non-binding fraction. Assays of the nonbound component were done in the presence of 0.15 mg of the retentate pool. Activities were determined at the end of a 30-min incubation.
on CHP reduction. As seen in Table III, sodium arsenite, iodoacetamide, and \(^{N}\)-ethylmaleimide all diminished the reductase activity, with \(^{N}\)-ethylmaleimide being the most potent inhibitor.

**Characterization of the 57-kDa Protein**—The spectrum of the purified 57-kDa component (Fig. 5A) has maxima at 280, 380, and 448 nm and a shoulder at 474 nm. The visible spectrum is characteristic of a flavoprotein, and the identity and stoichiometry of the cofactor were determined by trichloroacetic acid extraction and HPLC analysis (27, 28). The visible spectrum is characteristic of a flavoprotein, and the identity and stoichiometry of the cofactor were determined by trichloroacetic acid extraction and HPLC analysis (27, 28). Based on the quantities and retention times of the extracted flavin, the protein contains 1.1 mol of FAD/subunit. The presence of flavin, together with the finding that the protein binds to affinity resins like 5'-AMP agarose and Affi-Gel Blue, implies that the 57-kDa protein is the site of NAD(P)H interaction.

The \(^{NH_2}\)-terminal sequence for the first 25-amino acid residues was determined by sequential Edman degradation. However, searches of two protein data bases (Dayhoff and Genbank) gave no significant matches to known proteins.

\(^1\) The \(^{NH_2}\)-terminal sequences of the two proteins were determined using the Edman degradation method followed by HPLC analysis of the phenylthiohydantoin amino acids. The F52a sequence: MLDTNMTNLRAVLDRKLTKPV?LIA. The C22 protein sequence: GLIDTKIPFKNNAFHNGHFSVT.

**TABLE II**

| Added component | Conc. | % Activity relative to |  
|-----------------|------|------------------------|
| Protein additions\(^a\) | | |
| None | 0 | 100 |
| BSA | 10 | 900 |
| Apo-ferritin | 2.5 | 50 |
| Ovalbumin | 10 | 250 |
| Conalbumin | 10 | 580 |
| \(^{a}\)-Amylase | 5 | 40 |
| Thyroglobulin | 10 | 300 |
| Lysozyme | 10 | 100 |
| Other additions\(^b\) | | |
| None | 0 | 100 |
| Octyl glucopyranoside | 10 | 30 |
| Triton X-100 | 2 | 130 |
| Unilamellar vesicles | 0.5 | 130 |
| Multilamellar vesicles | 0.5 | 110 |
| Glycerol | 150 | 70 |
| BSA | 7.5 | >1300 |

\(^a\) Standard CHP reduction assays were performed as described under "Experimental Procedures" using 13 \(\mu\)g of the purified F52a protein and 55 \(\mu\)g of the pure C22 component along with an exogenous protein at the indicated final concentration. Assays were done in duplicate and the results are their average.

\(^b\) Experiment was performed as described above with the exception that 50 \(\mu\)g of component F52a and 130 \(\mu\)g of C22 were used. Vesicles were prepared from dl-myristoyl phosphatidylycholine by published protocols (25, 26).

**TABLE III**

| Inhibition of CHP reduction by thiol reactive reagents |  
|-----------------------------------------------------|
| Inhibitor | Concentration | Reduction rate | % Inhibition |
|-----------|---------------|----------------|-------------|
| None | | | 0 |
| NaAsO\(_2\) | 0.4 | 0.74 | 31 |
| | 4.0 | 0.29 | 73 |
| Iodoacetamide | 0.4 | 0.34 | 69 |
| | 4.0 | 0.14 | 87 |
| \(^{N}\)-Ethylmaleimide | 0.4 | 0.13 | 88 |
| | 4.0 | 0.06 | 94 |

This sequence information has since been used to identify the structural gene in \(S.\) typhimurium.\(^5\)

Since the mechanisms of many flavoprotein reductases involve a disulfide-dithiol cycle (29), cysteine content was measured by DTNB titration. The protein contains 2.2 mol of RSH/mol of F52a subunit. However, reduction with excess dithiothreitol or \(\beta\)-mercaptoethanol produces six additional DTNB reactive sulphydryls (Table IV). Anaerobic reduction of the protein by excess NADPH can also produce additional DTNB reactive sulphydryls (4.8) not present in the protein as isolated.

Gel permeation chromatography of the 57-kDa protein on a TSK-3000SW HPLC sizing column and on a Bio-Gel A0.5m column gave apparent molecular masses of 140,000 and 130,000, respectively (data not shown), suggesting that the
protein behaves as a dimer in solution. Two-dimensional gel electrophoresis (Fig. 4A) indicates that the protein has a pI of 6.9 (for reduced protein) while nonreduced isoelectric focusing gel electrophoresis suggests a value closer to 6.4 (not shown).

Following purification, the 57-kDa flavoprotein was assayed for its ability to reduce a number of electron acceptors including molecular oxygen, 2,6-dichloroindophenol, DTNB, methylene blue, nitro blue tetrazolium, and cytochrome c. All of these could be reduced with either NADPH or NADH as reduct donor. Reduction of both cytochrome c and nitro blue tetrazolium were inhibitable with superoxide dismutase, suggesting that these reactions are mediated by superoxide anion, produced via the oxidase reaction (data not shown). When compared with wild-type cells, crude extracts of oxyR1 cells exhibit higher levels of NAD(P)H diaphorase (2,6-dichloroindophenol reduction) and NAD(P)H oxidase (O2 reduction). These increases are probably due to the constitutive overproduction of the F52a protein.

In crude, cell-free extracts both NADPH and NADH will support CHP reduction. Following purification, a generating system for either NADPH or NADH (using Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase) works equally well in providing reducing equivalents for CHP reduction.

Characterization of the 22-kDa Protein—By contrast to the 57-kDa protein, the spectrum of the 22-kDa protein (Fig. 5B) displays no visible or UV chromophore other than the aromatic amino acid absorption centered at 278 nm. The NH2-terminal sequence of this component has also been determined for the first 25 amino acids6 and like the 57-kDa protein no match with proteins in the Dayhoff or Genbank data bases was found. As with the F52a protein, this information has proven useful for identifying the structural gene for the C22 protein.9 DTNB titration of the 22-kDa protein was also done (Table IV), since a sulfhydryl mechanism involving both proteins requires cysteines on both. Reaction of DTNB with purified 22-kDa protein liberated no free thiionitrobenzoate. Following pretreatment of the protein with excess dithiothreitol (or β-mercaptoethanol), titration gives a value of 1.9 ± 0.2 mol of thiol/mol of subunit.

Gel-filtration chromatography of the purified 22-kDa component gives an apparent molecular mass of 65,000 daltons when using a TSK-3000SW column. However, use of a Bio-Gel A0.5m column with the same protein gave an apparent value of 235,000 daltons. Inclusion of a reducing agent into the mobile phase of the HPLC sizing column resulted in the generation of a species with a molecular mass of approxi-

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**TABLE IV**

| Protein component—pretreatment | nmol RSH/nmol subunit |
|-------------------------------|-----------------------|
| C22 Native                    | 0.1 ± 0.03 (n = 3)    |
| β-Mercaptoethanol reduced     | 1.9 ± 0.08 (n = 3)    |
| Dithiothreitol reduced        | 1.8 ± 0.02 (n = 3)    |
| F52 Native                    | 2.1 ± 0.3 (n = 4)     |
| β-Mercaptoethanol reduced     | 7.6 ± 0.8 (n = 2)     |
| Dithiothreitol reduced        | 8.0 ± 0.1 (n = 2)     |
| NADPH reduced                 | 7.2 ± 0.4 (n = 2)     |

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* L. A. Tartaglia and B. N. Ames, unpublished results.

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**Fig. 5. UV visible spectra of the purified AHP reductase components.** The UV visible spectrum of each component (in 50 mM KPi, pH 7.0) was obtained with a Cary 219 spectrophotometer. The protein concentrations were for the F52a component (panel A) 0.75 mg/ml and for the C22 component (panel B) 0.93 mg/ml.

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mately 300,000 daltons. The extreme variability of the native molecular weight under different chromatographic conditions suggests that this component readily forms aggregates and prevents any conclusion about native molecular weight. Two-dimensional gel electrophoresis indicates an isoelectric point of 5.1.

**Alternate Substrates for the Alkyl Hydroperoxide Reductase**—Although the activity has been purified based on the ability to reduce a model peroxide (CHP), this is not a component with physiological significance for *S. typhimurium*. In order to assess a wider range of potential substrates, an assay was used which does not depend on the unique properties of CHP (hydrophobicity and UV absorbance). The reaction of the iodide anion with a wide variety of hydroperoxides to produce the colored tri-iodide anion has been extensively to analyze biological materials for the presence of oxidation products (10). Treatment of CHP with an NADPH-generating system and partially purified reductase results in the time-dependent disappearance of the iodide reactive material (Fig. 6). As expected, incubation in the absence of NADP gives no peroxide reduction. Fig. 6 also shows that two other peroxides (t-butyl hydroperoxide and ethyl hydroperoxide) are reduced at rates comparable to CHP. (For the partially pure oxyR1 extract used in these experiments, specific activities of 10–20 nmol/min/mg were obtained.) In addition to the three peroxides in Fig. 6, the following alkyl hydroperoxides are also substrates: benzyl hydroperoxide (I), p-methane hydroperoxide (a mixture of secondary monohydroperoxides) (II), and benzoyl hydroperoxide (III). The same broad specificity was also seen when the purified components were recombined and assayed by this method.

Of the hydroperoxides tested using this assay, the only one which was not a substrate was hydrogen peroxide. This is likely to be due to enzyme inactivation, since inclusion of an equivalent concentration of hydrogen peroxide in the standard
Alkyl Hydroperoxide Reductase in Escherichia coli—A mutant of \( E.\) coli K-12 has been isolated (oxyR2, TA4110) which is resistant to both hydrogen peroxide and alkyl hydroperoxides (4). As with \( \text{oxyRI} \), whole cells and crude extracts of \( \text{oxyR2} \) possess enhanced ability to reduce CHP. Using a slightly modified protocol, the two components of the alkyl hydroperoxide reductase were purified. The initial resolution of the fractions was effected using a 5'-AMP agarose column since the flavoprotein component of the \( E.\) coli enzyme, unlike that of \( S.\) typhimurium, does not bind efficiently to a blue dye affinity resin. Subsequent steps were identical to those described for the \( S.\) typhimurium proteins. During purification of the \( E.\) coli proteins, reconstitution of the activity was done with the complementary fraction derived from \( S.\) typhimurium extract. The success of such heterologous complementation indicates the basic similarity of the proteins derived from these two sources.

The \( E.\) coli and \( S.\) typhimurium 57-kDa proteins have the same molecular masses and similar isoelectric points (the pi of the \( E.\) coli protein is slightly more acidic) as determined by two-dimensional gels of cell extracts and of the purified species (data not shown). By these criteria, the two 22-kDa proteins appear to be the same. Hydroperoxide substrate specificity for the \( E.\) coli enzyme has not been determined.

Both flavoprotein components contain FAD and exhibit NAD(P)H oxidase and diaphorase activities in addition to their coupled hydroperoxide reductase activity. Aside from the minor differences between the proteins from the two organisms (i.e. their affinities to 5'-AMP-agarose and in their isoelectric points), the most significant difference concerns their regulation. In \( S.\) typhimurium, the 57-kDa protein appears to be both heat-shock and hydrogen peroxide-inducible; however, the \( E.\) coli equivalent is only induced by peroxides (5).

**DISCUSSION**

While many products of oxygen radical damage are directly toxic, alkyl hydroperoxides may be especially detrimental because of their ability to initiate and propagate free-radical chain reactions leading to DNA and membrane damage (30, 31). To understand how bacteria defend against oxidative damage by alkyl hydroperoxides, we have studied a \( S.\) typhimurium mutant (\( \text{oxyRI} \)) that is resistant to mutagenesis and killing by both hydrogen peroxide and alkyl hydroperoxides (4, 5, 32). We have purified and characterized two proteins which together reduce alkyl hydroperoxides and which correspond to two of the nine proteins which are overexpressed by the \( \text{oxyRI} \) mutant.

Mammalian cells contain the flavoprotein glutathione reductase and the selenoenzyme glutathione peroxidase to defend against damage from alkyl hydroperoxides. These two proteins together catalyze the NADPH-dependent reduction of a wide range of alkyl hydroperoxides (33, 34). Recently Ursini et al. (35, 36) have characterized a second mammalian GSH peroxidase which is only active toward phospholipid hydroperoxides. The presence of GSSG reductase in \( E.\) coli is well documented (29, 37, 38). It was therefore surprising, given the important protective function of the peroxidase, when it was reported that bacteria lack GSH peroxidase activity (39). Our work demonstrates that bacteria contain a novel glutathione-independent alkyl hydroperoxide reductase activity which may serve an equivalent role in protecting against alkyl hydroperoxides.

Both the GSH-dependent system and AHP reductase require the presence of two separable protein components: a dimeric FAD protein with subunit molecular mass of about 55,000 and a second 22,000 protein. Many other characteristics of the two systems differ. Glutathione reductase isolated from bacteria and mammals is a flavoprotein, utilizes NADPH as electron donor, and lacks oxidase, diaphorase, and DTNB reductase activities (29). By contrast, the flavoprotein component of the alkyl peroxide reductase can use either NADH or NADPH as the reducing agent for a wide variety of substrates including \( \text{O}_2\), 2,6-dichloroindophenol, methylene blue, DTNB, and in the presence of the 22-kDa protein, alkyl hydroperoxides. Glutathione peroxidase (23 kDa) is known to contain covalently bound selenium at the active site (34). However, when \( S.\) typhimurium is grown in the presence of radioactive selenium, no label is incorporated into 22-kDa.
protein. The distinction between these two systems is further accentuated by the fact that a strain containing a mutation in GSSG reductase (gorA) has a normal rate of CHP reduction when assayed in whole cells (data not shown).

Two other bacterial redox systems have been described which can use peroxides as substrates in GSH-independent reactions. In Pseudomonas, hydroxylation of hydrocarbons and fatty acids is catalyzed by three proteins, an alkane hydroxylase, rubredoxin, and rubredoxin reductase. The latter two components are an iron-sulfur redox protein and its flavoprotein reductase (1 FAD/55-kDa subunit). In the absence of the hydroxylase these can promote NADH-dependent reduction of a wide range of alkyl hydroperoxides, which may be chemical intermediates in the conversion of alkane to alcohol at 40 (41).

In bacteria which lack de novo heme biosynthesis (such as Streptococcus and Lactobacillus), the flavoprotein NADH peroxidase catalyzes the destruction of hydrogen peroxide normally done by catalase. The most carefully studied enzyme was isolated from Streptococcus faecalis by Dolin (42–45). Similar activities have also been described in S. lactis (46, 47), Lactobacillus plantarum (48), and L. casei (49, 50). The S. faecalis enzyme contains 1 FAD/60,000-dalton subunit, and this dimeric protein is solely responsible for NADH-dependent reduction of hydrogen peroxide (42–45). In addition, the enzyme can catalyze reduction of ferricyanide and menadione. However, O2, 2,6-dichlorophenolindophenol, and a variety of dyes are not reduced at significant rates (44). Recent experiments of Claibourne and Poole have elucidated the detailed redox properties of the enzyme (51, 52). The protein is now commercially available (from Sigma), and our preliminary results demonstrate that CHP and t-BHP are not substrates for this enzyme.5

For all their differences, one similarity of these diverse peroxide reductase systems is most striking: all are dependent on FAD-containing proteins of 50–60 kDa. Recent work from a number of sources demonstrates the high degree of sequence homology between the active site peptides of several FAD-containing oxidoreductases, including GSSG reductase, lipooxidase dehydrogenase, trypanothione reductase, and curvure reductase (29, 53–55). In addition to their subunit molecular weights and cofactor, these enzymes also all use catalytic cystine residues, a feature which may be shared by the flavoprotein component of the alkyl hydroperoxide reductase.

As shown above, two of the sulphydryls on the F52a protein can be titrated by DTNB and the reduction of CHP can be inhibited by thiol reactive reagents like N-ethylmaleimide. Sequence determination of the recently cloned F52a gene6 will allow examination for such a conserved active site peptide.

Such a structural homology could have mechanistic as well as evolutionary implications. The presence of this conserved active site would strongly suggest that the catalytic cycle involves the transfer of reducing equivalents from the dihydropyrimidine cofactor to the FAD and then to a nearby redox active disulfide. The requirement for a second protein (C22), which also contains a redox active disulfide, leads us to propose the mechanism summarized in Scheme 1. In this model, the site of substrate peroxide reduction is on the F52a protein. Coupling of NAD(P)H oxidation to peroxide reduction is mediated by electron transfer between a redox active dithiol on the F52a protein and the disulfide on C22.

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