Flavin-Dependent Alkyl Hydroperoxide Reductase from *Salmonella typhimurium*. 1. Purification and Enzymatic Activities of Overexpressed AhpF and AhpC Proteins[†]

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ABSTRACT: The two components, AhpF and AhpC, of the *Salmonella typhimurium* alkyl hydroperoxide reductase enzyme system have been overexpressed and purified from *Escherichia coli* for investigations of their catalytic properties. Recombinant proteins were isolated in high yield (25−33 mg per liter of bacterial culture) and were shown to impart a high degree of protection against killing by cumene hydroperoxide to the host *E. coli* cells. We have developed quantitative enzymatic assays for AhpF alone and for the combined AhpF/AhpC system which have allowed us to address such issues as substrate specificity and inhibition by thiol reagents for each protein. All assays gave identical results whether overexpressed *S. typhimurium* proteins from *E. coli* or proteins isolated directly from *S. typhimurium* were used. Anaerobic hydroperoxide reductase assays have demonstrated that cumene hydroperoxide, ethyl hydroperoxide, and hydrogen peroxide can all be reduced by the combined enzyme system. AhpF possesses multiple pyridine nucleotide-dependent activities [5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reductase, oxidase, transhydrogenase, and, in the presence of AhpC, peroxide reductase activities]. Although AhpF can use either NADH or NADPH as the electron donor for these activities, NADH is the preferred reductant ($K_{m,app}$ of AhpF for NADH was more than 2 orders of magnitude lower than that for NADPH when analyzed using DTNB reductase assays). Thiol-modifying reagents react readily with each reduced protein, leading to complete loss of hydroperoxide and DTNB reductase activities. In contrast, thiol modification of reduced AhpF does not affect transhydrogenase or oxidase activities. These data provide the first direct evidence for a catalytic mechanism for peroxide reduction involving redox-active disulfides within each protein.

Protection of aerobic organisms from the toxic and damaging effects of oxygen is conferred by a wide variety of enzymes which, in bacterial systems at least, can be coordinately regulated through redox-sensitive transcriptional factors (Storz et al., 1990; Farr & Kogoma, 1991; Shigenaga et al., 1994; Sies, 1991). Using bacterial mutants altered in their OxyR-regulated stress responses, Ames and co-workers identified a glutathione-independent peroxidase system in *Escherichia coli* and *Salmonella typhimurium* with a protective role in limiting oxygen-linked DNA damage (Christman et al., 1985; Storz et al., 1987). Subsequent studies allowed identification of the two proteins which comprise this peroxidase system as a 57 kDa flavoprotein (AhpF, formerly designated F52a) and a 21 kDa protein lacking a chromophoric cofactor (AhpC, formerly designated C22). Although AhpF and AhpC were readily separated during purification, both were required for the catalysis of NADH- and NADPH-dependent reduction of a wide variety of hydroperoxide substrates to their corresponding alcohols.

Following the cloning of the *ahpCF* locus (Storz et al., 1989; Tartaglia et al., 1990), inspection of the deduced amino acid sequence of AhpF allowed clear identification of this flavoprotein as the second known member of the thioredoxin reductase (TrR)[1] branch of the flavoprotein pyridine nucleotide:disulfide oxidoreductase enzyme family. Although sequence alignments do not indicate any comparable relationship between AhpC and thioredoxin (Tr), homologues of AhpC now appear to be extremely widespread throughout all kingdoms (Chae et al., 1994b). Inspection of multiple sequence alignments performed on AhpF and AhpC homologues indicates conservation of the two half-cystine residues within AhpF which correspond to the known active-site cysteinyl residues of TrR and of the two half-cystine residues of AhpC present in virtually all homologues identified (Chae et al., 1994b). This observation provides support for the hypothesis that redox-active cystine disulfides are involved in catalysis of hydroperoxide reduction by the AhpF and AhpC proteins (Jacobson et al., 1989).

Although a major portion of AhpF aligns well with the *E. coli* TrR sequence and shows 35% amino acid identity, there is an extra N-terminal region within AhpF of about 200 amino acids which has no counterpart in TrR (Chae et al., 1994b; Tartaglia et al., 1990). Most bacterial AhpF homologues identified to date also include this N-terminal region, including two flavoproteins which were identified as hydro-

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[1] Abbreviations: TrR, thioredoxin reductase; Tr, thioredoxin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoate; NTSB, 2-nitro-5-thiosulfobenzoate; IPTG, isopropyl β-D-thiogalactopyranoside; DMSO, dimethyl sulfoxide; AcPyADH, reduced 3-acetylpyridine adenine dinucleotide; $AcPyAD^+$, oxidized 3-acetylpyridine adenine dinucleotide; LB, Luria-Bertani medium; GuHCl, guanidine hydrochloride; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate.

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gen peroxide-producing NADH oxidases [NADH oxidases from *Amphibacillus xylanus* (Niimura et al., 1993) and *Streptococcus mutans* (Higuchi et al., 1994)] and a third flavoprotein identified as an NADH dehydrogenase primarily on the basis of its diaphorase activity [NADH dehydrogenase from *Bacillus alcalophilus* (Xu et al., 1991)]. The location of partial open reading frames homologous with *S. typhimurium ahpC* upstream of all three of the structural genes of these flavoproteins, similar to the arrangement of the *S. typhimurium ahpCF* structural genes, suggests that these bacterial flavoproteins can also function as reductases in an alkyl hydroperoxide reductase system (Chae et al., 1994b). Recent evidence has confirmed the ability of the *A. xylanus* NADH oxidase to replace AhpF in catalyzing NADH-dependent hydroperoxide reduction in the presence of *S. typhimurium* AhpC (Niimura et al., 1995).

In these studies, we have undertaken a detailed definition of the catalytic activities of *S. typhimurium* AhpF and AhpC. Our studies have been facilitated by our ability to overexpress and isolate the recombinant proteins from *E. coli* in high yields. We report catalytic effects of different pyridine nucleotide substrates, thiol-modifying reagents, and ionic strength variation using several different assay systems.

EXPERIMENTAL PROCEDURES

Materials. NADH and NADPH were purchased from Boehringer-Mannheim. Sigma was the supplier of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), $AcPyAD^+$, *Naja naja* venom phosphodiesterase, dimethyl sulfoxide (DMSO), streptomycin sulfate, cumene hydroperoxide (80%), and molecular biology-grade ammonium sulfate. Organic solvents including methanol, acetic acid, and ether were purchased from Fisher; Baker-analyzed methanol was used as solvent for HPLC. Hydrogen peroxide (30%) was from Fisher; DTNB, SDS, ultrapure urea, ultrapure glycine, and other buffer components were obtained from Research Organics, Inc. Bacteriological media components were from Difco. Ethyl hydroperoxide was obtained from Polysciences, Inc. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and used as recommended by the manufacturer. New England Nuclear was the supplier for [α-^{35}S]dATP.

Bacterial Strains and Culture Procedures. E. coli XL1-Blue (Stratagene) and JM109(DE3) were used as plasmid hosts. Cultures were stored at −80 °C in Luria-Bertani (LB) broth containing 15% glycerol and plated fresh on LB agar plates containing the appropriate antibiotic prior to inoculation of liquid cultures. Chloramphenicol (25 μg/mL) or ampicillin (50 μg/mL) (from 1000× stocks prepared in 100 and 50% ethanol, respectively) was included as required. Liquid cultures were inoculated from a single colony, grown first in 4 mL of LB broth containing the appropriate antibiotic, and then used as 1 or 2% inocula for larger cultures. For protein expression, cultures were grown to an A_{600} value of 0.9 and then induced for protein expression by addition of IPTG to a final concentration of 0.4 mM. Induced cultures were allowed to continue growing for 3 h prior to harvest.

Expression and Purification of Recombinant AhpC and AhpF. For improvements in protein expression and hydroperoxide sensitivity testing, *ahpC*, *ahpF*, and the entire *ahpCF* locus were subcloned into pOXO4 as depicted in Figure 1, yielding the expression plasmids pAC1, pAF1, and pACF1, respectively. pOXO4 is a T7 RNA polymerase-dependent expression plasmid derived from pSP73 (Promega), with modifications made to allow selection by chloramphenicol rather than ampicillin resistance and to include a T7 transcription terminator on the side of the multiple cloning site opposite from the T7 promoter (Parsonage et al., 1993). pAQ27, a pUC18 derivative with a 4.1 kb insert containing the *ahpCF* gene region and flanking sequences, was generously provided by Dr. Gisela Storz (Tartaglia et al., 1989). DNA isolation and manipulation procedures involved use of the GeneClean kit (Bio101) for isolation of DNA from agarose gels or enzyme incubation mixtures, preparation of competent cells using the TSS method (Chung et al., 1989), sequencing of plasmid constructs by the Sanger method as outlined in the USB Sequenase 2.0 kit, and other standard procedures for subcloning DNA fragments (Ausubel et al., 1992; Sambrook et al., 1989).

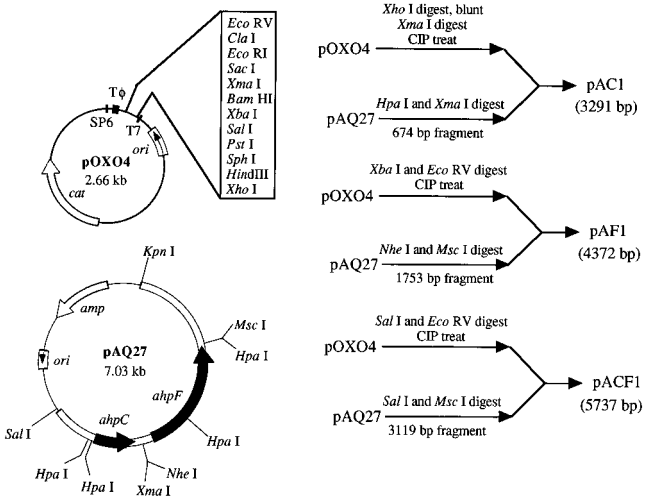

FIGURE 1: Construction of expression plasmids for AhpC and AhpF. The expression plasmid for AhpC, denoted pAC1, was constructed by ligation of the *Hpa*I-*Xma*I fragment from pAQ27 into pOXO4 digested first with *Xho*I, blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I and 0.25 mM of each of the four dNTPs, isolated from an agarose gel using GeneClean (Bio 101), redigested with *Xma*I, and then dephosphorylated with calf intestinal alkaline phosphatase. In each case, DNA fragments were isolated with GeneClean prior to overnight ligation with T4 DNA ligase, followed by transformation of the ligated DNA into competent XL1-Blue cells. The pAF1 plasmid for AhpF expression and the pACF1 plasmid containing coding regions for both proteins were constructed as shown using techniques similar to those for pAC1 construction.

The protocol developed for the purification of AhpC included several significant changes relative to the previously reported procedure (Jacobson et al., 1989), starting with pelleted bacteria from 8 L of an IPTG-induced culture of JM109(DE3) bacteria harboring the pAC1 plasmid. The standard buffer used in all procedures was 25 mM potassium phosphate (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), unless otherwise indicated. Cell extract was prepared using a BioSpec Products Bead-Beater (six cycles of 90 s each, with intervening 90 s periods for cooling) and several centrifugation and washing steps (18000*g*, 20 min each) to remove cell debris and glass beads. Streptomycin sulfate was added to a final concentration of 2.5% (w/v) to precipitate nucleic acids, followed by stirring for 30 min at 4 °C and centrifugation at 23000*g* for 20 min. The supernatant was subjected to 20 and 60% ammonium sulfate treatments and centrifugation essentially as described

previously (Jacobson et al., 1989). The pelleted protein from the 60% ammonium sulfate treatment was resuspended in standard buffer containing 20% ammonium sulfate for direct loading onto a 37 × 2.5 cm Phenyl Sepharose 6 Fast Flow Column (Pharmacia LKB Biotechnology Inc.) preequilibrated with 20% ammonium sulfate in standard buffer. The column was washed with 20% ammonium sulfate, and AhpC was eluted by a linear gradient from 20 to 0% ammonium sulfate (700 mL total volume). An additional wash of the standard buffer without ammonium sulfate was required to completely elute the protein. Fractions containing 80−90% of the single large peak of protein (as assessed by A_{280}) were pooled and dialyzed against two 6 L changes of the standard buffer. The dialyzed protein was loaded onto a 37 × 2.5 cm DEAE−cellulose column (Whatman DE52) preequilibrated with 60 mM potassium phosphate (pH 7.0) containing EDTA and then eluted with a linear gradient from 60 to 140 mM potassium phosphate (1 L total volume). Fractions judged to be pure by analysis on SDS−polyacrylamide gel electrophoresis (SDS−PAGE) were pooled, dialyzed against standard buffer, aliquoted, and frozen at −20 °C until needed.

Purification of AhpF followed a protocol similar to that previously reported (Jacobson et al., 1989). Crude extract from 8 L of a culture of JM109(DE3) harboring the pAF1 plasmid was obtained, and nucleic acids were precipitated as described above for AhpC. In our modified procedure, the pelleted protein from the second ammonium sulfate precipitation was resuspended and dialyzed against 20 mM potassium phosphate (pH 7.0) containing 1 mM EDTA, applied to a DEAE−cellulose column (DE52) equilibrated in the same buffer, and then washed and eluted with a linear gradient (1 L total) from 20 to 80 mM potassium phosphate at pH 7.0. For this and the following column, fractions with an A_{280}/A_{450} ratio of 6.1 or less were pooled. The pool from the DE52 column was loaded directly onto the Affi-Gel Blue column (Bio-Rad), washed with the standard buffer, and then eluted with a 1 L linear gradient starting with standard 25 mM potassium phosphate buffer and ending with 0.6 M potassium phosphate at pH 7.0 containing 0.3 M NaCl and 1 mM EDTA (Jacobson et al., 1989). Pure fractions from this column as assessed by SDS−PAGE were pooled, aliquoted, and stored at −20 °C until needed.

Purification of AhpF and AhpC proteins from *S. typhimurium* followed essentially the same chromatographic methods as those described above for the recombinant proteins, starting with bacterial extract from 45 or 60 L of *oxyR1* culture (Jacobson et al., 1989). *oxyR1* (also designated TA4100) is a *S. typhimurium* strain bearing a mutation in *oxyR* which results in constitutively high transcriptional activity of the gene product, OxyR, which activates *ahpCF* transcription (Christman et al., 1985, 1989). Two additional columns, BioGel A-0.5m and AMP-agarose, were necessary for satisfactory purification of the natural AhpF protein, and the purification of AhpC also included passing the pool from the DE52 column through an equilibrated Affi-Gel Blue column to remove contaminating AhpF (Jacobson et al., 1989).

Survival of AhpF- and/or AhpC-Expressing Bacteria Exposed to Cumene Hydroperoxide. Liquid cultures (50 mL) of JM109(DE3) harboring pOXO4, pAC1, pAF1, or pACF1 plasmids were induced for 1 h with 0.4 mM IPTG added after growth to $A_{600} = 0.8$ and then exposed for 10 min at room temperature to varying concentrations of cumene hydroperoxide diluted into DMSO (1 mL of culture was added to 50 μL of cumene hydroperoxide, DMSO, and water containing a maximum of 16 μL of DMSO). Following cumene hydroperoxide/DMSO treatment, pelleted bacteria were washed once with LB, then resuspended into the same volume of LB, diluted through 10-fold serial dilutions (50 μL of bacteria was added to 450 μL of LB), and plated on chloramphenicol-containing LB agar plates. DMSO was shown to have no effect on colony count; percentages of surviving bacteria were determined by comparison between peroxide-treated and DMSO-treated colony counts. Statistical analyses were carried out using *t*-test comparisons at each concentration, 0.1 and 0.2 mM, of cumene hydroperoxide used.

Protein Chemical Methods and N-Terminal Amino Acid Sequence Determinations. Identification of the flavin coenzyme released on precipitation of the natural and recombinant AhpF proteins with 5% trichloroacetic acid was carried out by HPLC using isocratic elution with 15% methanol combined with an aqueous buffer at pH 7.0 (giving a final concentration of 4.25 mM potassium phosphate) and a 250 × 4.6 mm Supelcosil LC-18 column [conditions were based on those reported by Light et al. (1980)]. Under these conditions, FMN and FAD standards eluted at 13.4 and 17.0 min, respectively. Further analysis of the released FAD cofactor was carried out through the conversion of FAD (both the standard and protein-derived coenzyme) to FMN on treatment with *N. naja* venom phosphodiesterase. FAD content of the natural and recombinant AhpF proteins was assessed by comparison of spectra of the enzymes denatured in 4 M GuHCl with native enzyme spectra and with an FAD standard in 4 M GuHCl. Protein concentrations were assessed by the microbiuret method using bovine serum albumin as the standard (Bailey, 1962; Janatova et al., 1968). SDS−PAGE of 10% slab gels was performed as described (Bollag & Edelstein, 1991) using a Gibco-BRL vertical slab gel apparatus. Protein bands were stained with Coomassie Blue R-250 and destained in methanol/acetic acid/water (3:1:6).

For N-terminal sequence analyses, 1 nmol of each recombinant protein was concentrated and rediluted twice with deionized water in CM-30 Centricons (Amicon) to significantly reduce the phosphate buffer concentration prior to direct sequence analyses. Amino acid sequence analyses were performed in the Protein Analysis Core Laboratory of the Cancer Center of Wake Forest University supported in part by NIH Grants CA-12197 and RR-04869, as well as by a grant from the North Carolina Biotechnology Center.

Spectral and Anaerobic Experiments. A thermostatted Milton Roy Spectronic 3000 diode array spectrophotometer with 0.35 nm resolution was used to collect all absorbance spectra and to carry out all anaerobic absorbance measurements (kinetic data were collected at 1 s intervals). Aerobic assays following absorbance changes at a single wavelength were carried out using a thermostatted Gilford 220 updated recording spectrophotometer with a DU monochromator. Kinetic studies of DTNB reductase activities of AhpF were performed using an Applied Photophysics DX.17MV stopped-flow spectrophotometer thermostatted at 25 °C. Extinction coefficients used were as follows (in M^{-1} cm^{-1}): FAD, 11 300 at 450 nm; NADH, 6 220 at 340 nm; NADPH, 6 200 at 340 nm; NAD^+, 17 800 at 259 nm; AcPyADH, 9 100 at 363 nm; $AcPyAD^+$, 16 400 at 260 nm (*Pabst Laboratories Circular*, 1977); and TNB, 14 150 at 412 nm (Riddles et al., 1979). Hydrogen peroxide and ethyl hydroperoxide con-

centrations were determined by assay with horseradish peroxidase and *o*-dianisidine, using a molar extinction coefficient of 11 300 M^{-1} cm^{-1} at 460 nm for the oxidized product (*Worthington Enzyme Manual*, 1972).

Activity Assays and Incubations with Thiol Reagents. Anaerobic peroxidase assays following NADH oxidation at 340 nm were carried out at 25 °C in a total volume of 1.0 mL containing 300 μM NADH, 1 mM cumene hydroperoxide, ethyl hydroperoxide, or hydrogen peroxide, and 50 mM potassium phosphate at pH 7.0, with 100 mM ammonium sulfate, 0.5 mM EDTA, and 0.01−5 nmol of both AhpF and AhpC (optimal activity for each protein was seen in potassium phosphate buffer at pH 7.0 relative to pH 6.5 or 7.5 and higher pH Tris buffers). Cumene hydroperoxide was diluted 50-fold into DMSO prior to addition to the assay mixture. For AhpF-dependent peroxidase assays, 5 nmol of AhpC was incubated with buffer components and pyridine nucleotide at room temperature with repeated evacuation and nitrogen flushing (at least ten cycles over 20 min), then transferred to a water bath at 25 °C for at least 5 min, mixed with 10−75 pmol of AhpF in one sidearm (the change in A_{340} was observed for 1 min to evaluate the background rate), and finally mixed with the hydroperoxide substrate to record the rate of A_{340} loss. AhpC-dependent peroxidase assays were carried out in a similar fashion, except that substrates and buffer components were incubated in the main chamber and 5 nmol AhpF and 100−200 pmol of AhpC in the sidearms during anaerobiosis. In the latter case, turnover in the presence of only AhpF was assessed prior to addition of AhpC.

Standard assays for the DTNB reductase activity of AhpF (similar to those used to analyze TrR/Tr system activities; Prongay et al., 1989) were carried out at 25 °C using 1.0 mL of a solution containing a saturating concentration (50 μM) of NADH, 500 μM DTNB, 50 mM Tris-HCl at pH 8.0, 0.5 mM EDTA, and 3−12 pmol of AhpF. Rates of TNB production were followed at 412 nm and 25 °C, and the small spontaneous rate in the absence of protein was subtracted.

Standard transhydrogenase assays (Mulrooney & Williams, 1994) were carried out at 25 °C in 1 mL of solution containing 150 μM each of NADH and $AcPyAD^+$, 50 mM Tris-HCl at pH 8.0, 0.5 mM EDTA, 100 mM ammonium sulfate, and 10−20 pmol of AhpF (our studies indicated that loss of NADH and concomitant gain of the same amount of AcPyADH resulted in an increased absorbance at 390 nm with an extinction coefficient of 4080 M^{-1} cm^{-1}). Oxidase assays were performed at 25 °C in 1 mL of air-saturated buffer containing 150 μM NADH, 50 mM potassium phosphate at pH 7.5, 0.5 mM EDTA, and 30−60 pmol of AhpF, monitoring absorbance changes at 340 nm.

Inhibition of AhpF by thiol-directed reagents was examined by anaerobic preincubation in standard buffer of 700 nM AhpF with varying concentrations of inhibitor, in the presence or absence of 105 μM NADH. Preincubated AhpF protein was added directly to the DTNB reductase assay mixture to give a 300-fold or greater dilution of inhibitor. Of the thiol reagents used, sodium arsenite and *N*-ethylmaleimide were standardized by reaction with DTT prior to DTNB assay for free thiols, while iodoacetamide was standardized by direct reaction with TNB (made from DTNB plus limiting DTT) as assessed by the decrease in A_{412}.

To measure the effects of thiol reagents on AhpF-dependent peroxidase, oxidase, and transhydrogenase activities, 2.5 μM AhpF was preincubated with 25 μM *N*-ethylmaleimide in the presence or absence of 105 μM NADH and then diluted into the respective assay mixtures to reduce the *N*-ethylmaleimide concentration to 1.5 μM or lower. AhpC (5 μm) was preincubated with AhpF (50 nM) and inhibitor (25 μM) in the presence or absence of NADH (105 μM) for 30 min directly in the sidearm of the anaerobic cuvette set up for the standard AhpC-dependent peroxidase assay. Assays for the remaining AhpC-dependent peroxidase activity contained the indicated inhibitor diluted to 0.75 μM.

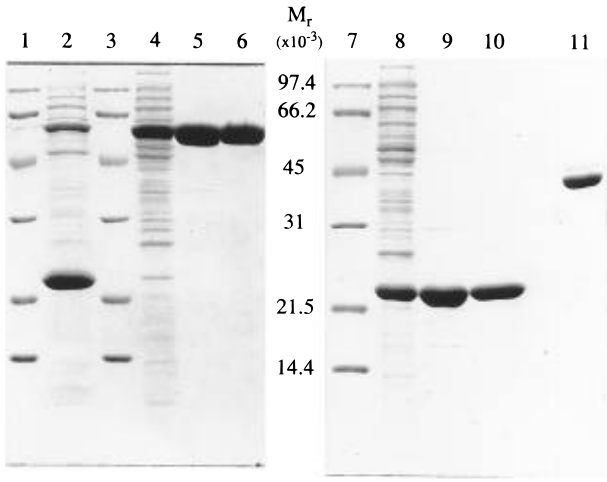

FIGURE 2: SDS−polyacrylamide gel electrophoresis of recombinant *S. typhimurium* alkyl hydroperoxide reductase proteins expressed in *E. coli*, at various stages of purification. Acrylamide gels (10%) were run with samples pretreated by boiling for 3 min in sample buffer containing 2% SDS. Samples in lanes 1−10 also included 5% 2-mercaptoethanol. The 0.75 mm thick gels were electrophoresed at 35 mA each in the presence of 1% SDS. Lanes 1, 3, and 7 contain low-range protein standards from Bio-Rad with indicated M_r values. Lanes 2, 4, and 8 contain 25 μg samples of the crude *E. coli* extracts from IPTG-induced JM109(DE3) harboring the pACF1, pAF1, and pAC1 overexpression plasmids, respectively. Lanes 5 and 6 contain 10 μg samples obtained during the purification of AhpF, following DEAE−cellulose and gel filtration chromatography, respectively. Similarly, lanes 9 and 10 contain samples from an AhpC purification, following phenyl sepharose and DEAE−cellulose chromatographic steps. Lane 11 contains 5 μg of the same sample applied to lane 10, but prepared under nonreducing conditions.

RESULTS

Expression and Purification of Alkyl Hydroperoxide Reductase Proteins, AhpF and AhpC. To obtain recombinant proteins in high yield, *ahpCF*-containing DNA fragments were subcloned into the pSP73-derived expression vector, pOXO4 (Figure 1; Parsonage et al., 1993). This system, which is based on T7 RNA polymerase-dependent expression, allows for the individual overexpression of each protein component (AhpF and AhpC) of the alkyl hydroperoxide reductase system and purification of each component to homogeneity (Figure 2). As described in Experimental Procedures, individual purification of AhpC and AhpF proteins from 8 L of LB chloramphenicol inoculated with pAC1- or pAF1-harboring JM109(DE3) resulted in yields of 260 and 200 mg of each pure protein, respectively. Enzymatic analyses of samples taken at the beginning and end of each purification (anaerobic AhpC-dependent peroxidase assays for AhpC and aerobic DTNB reductase assays for AhpF, see below) indicated that AhpC accounted for 23−45% of the total protein in the initial cell extracts and gave purified protein yields of 50−70%, while AhpF was about 17−24% of the total protein and gave yields of 70−85%.

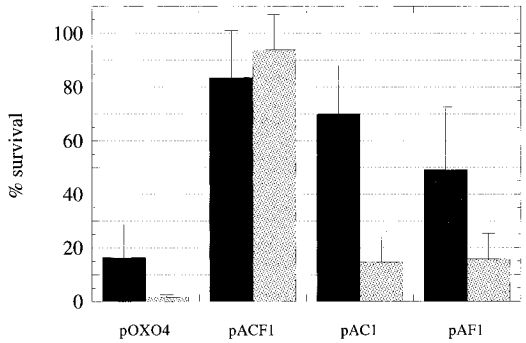

FIGURE 3: *In vivo* activity of recombinant alkyl hydroperoxide reductase proteins in protection against cumene hydroperoxide toxicity. JM109(DE3) *E. coli*-harboring expression vectors for each (pAC1 for AhpC and pAF1 for AhpF) or both (pACF1) alkyl hydroperoxide reductase proteins, or the parent vector itself (pOXO4), were grown in liquid culture, induced with IPTG for 1 h, and then subjected to 0.1 mM (shaded boxes) or 0.2 mM (dotted boxes) cumene hydroperoxide treatment for 10 min. Percent survival was evaluated by plating dilutions of the washed bacteria on antibiotic-containing selective media.

The high degree of purity achieved and the high yields of AhpC and AhpF proteins obtained represented a tremendous improvement compared with those of the constitutively expressed proteins isolated from the *S. typhimurium* mutant *oxyR1*, particularly in the case of the AhpF protein (Jacobson et al., 1989). Relative mobilities of the purified proteins on SDS−polyacrylamide gels (Figure 2) gave apparent molecular masses for AhpC and AhpF of 23 and 58 kDa, respectively (dimer of AhpC, seen under nonreducing conditions in lane 11, appears at an apparent molecular mass of 40 kDa).

Protection by Overexpressed Alkyl Hydroperoxide Reductase Proteins against Cumene Hydroperoxide Toxicity. In order to help assess the requirement *in vivo* for each of these protein components of alkyl hydroperoxide reductase to confer protection on host bacteria exposed to cumene hydroperoxide, cultures of JM109(DE3) overexpressing each or both protein(s) were compared in cumene hydroperoxide survival assays with those harboring the vector only. As shown in Figure 3, maximal protection is conferred on overexpression of both AhpC and AhpF (50% survival of pACF1-harboring cells is seen on the cumene hydroperoxide concentration being raised to about 1 mM, data not shown). Treatment with 0.1 mM cumene hydroperoxide gives results for all three constructs which show greater protection conferred relative to the vector control ($p < 0.01$ for pAF1 *vs* pOXO4; $p < 0.001$ for pACF1 and pAC1 *vs* pOXO4). Each protein, individually overexpressed, is less effective than the two proteins coexpressed in protecting these bacteria from cumene hydroperoxide-mediated killing; both pAC1 and pAF1 vectors conferred less protection toward treatment by 0.2 mM cumene hydroperoxide than did pACF1 ($p < 0.02$). In each case, endogenous levels of the *E. coli* alkyl hydroperoxide reductase protein(s) not encoded within the construct may be the limiting factor in conferring the partial protective behavior seen for the individually overexpressed proteins. Hydrogen peroxide-mediated killing is also reduced in these overexpressing bacteria, but the extent of protection conferred by these proteins is significantly less (data not shown).

Comparison of the Structural and Spectral Properties of Recombinant and Natural Alkyl Hydroperoxide Reductase Proteins. N-terminal sequences obtained for the first 32 and 22 amino acid residues of recombinant AhpC and AhpF proteins, respectively, were identical with those predicted from the DNA sequences of the *ahpC* and *ahpF* genes (Chae et al., 1994b; Jacobson et al., 1989). As previously shown, the initiating formylated methionine of AhpC is removed to give the mature protein, which starts with Ser (codon 2) at the N-terminus.

Purified recombinant alkyl hydroperoxide reductase proteins were identical in all ways tested with proteins purified directly from cell extracts of *oxyR1* (TA4100), a constitutively overexpressing strain of *S. typhimurium* (Christman et al., 1985; Jacobson et al., 1989), except that analyses of the natural form of AhpF isolated from *S. typhimurium* showed a variation in FAD content (Table 1; see below). The chromatographic behavior of each recombinant protein was essentially identical with that of its natural counterpart on each column used for the purification. Analyses of spectroscopic properties of AhpC proteins confirmed the absence of any chromophoric cofactor and allowed determination of the molar extinction coefficient for AhpC at 280 nm. Given a value of 1.18 for the absorption of a 1 mg/mL solution of AhpC at 280 nm and a calculated molecular mass of 20 616 Da for AhpC (excluding the N-terminal methionine which is removed), the molar extinction coefficient at 280 nm is 24 300 M^{-1} cm^{-1}.

Analyses of spectral properties and cofactor contents of AhpF proteins, on the other hand, indicated that the flavin content of the AhpF protein purified from *S. typhimurium* was more variable. Only about 10 mg of highly purified AhpF was isolated from 45 L of *S. typhimurium* culture in these cases. Our first preparation of purified enzyme gave a flavin content of 0.45 FAD/subunit, compared with 0.92 FAD/subunit for the recombinant enzyme; this difference was reflected in the much higher ratio of A_{280}/A_{450} for the *S. typhimurium*-purified AhpF (Table 1). A second purification of AhpF carried out under apparently identical conditions yielded protein with a flavin content of 0.87 FAD/subunit. This preparation was used in comparisons of enzymatic activity, as described below. Although the FAD-to-protein ratio within the natural AhpF protein was substoichiometric in one of the two preparations, the extinction coefficients of the protein-bound FAD at 450 nm for all preparations of AhpF were identical, 13 100 M^{-1} cm^{-1} (Table 1). Recombinant AhpF has ratios of 6.7, 5.6, 0.97, and 1.0 for absorbances at 273, 280, 380, and 450 nm, respectively. The absorbance at 280 nm of 1.19 for a 1 mg/mL solution and the calculated molecular mass of 56 735 Da for AhpF (assuming one FAD per subunit) give a value of 67 500 M^{-1} cm^{-1} for the molar extinction coefficient of the holoenzyme.

NADH-Dependent Peroxidase Assays of Each Protein, AhpF and AhpC. In order to carefully analyze the catalytic function of each protein within an NADH-dependent peroxidase assay, anaerobic assays were carried out with saturating substrates (300 μM NADH and 1 mM H_2O_2, ethyl hydroperoxide or cumene hydroperoxide) and both proteins, AhpF and AhpC. In order to obtain specific activities of either protein, an excess of the other protein is included, so that peroxidase assays are denoted as AhpF-dependent (with AhpF in limiting amounts and AhpC in excess) or AhpC-dependent (AhpC limiting and AhpF in excess). Under these conditions, each initial rate was linearly dependent on the amount of limiting protein added. This method has two distinct advantages over the previously used HPLC assays. First, it permits the direct and continuous measurement of

Table 1: Characteristics of Natural and Recombinant *S. typhimurium* Alkyl Hydroperoxide Reductase Proteins

characteristic	AhpF		AhpC	
	natural	recombinant	natural	recombinant
peroxide reductase activity (min^{-1})[a,b]				
cumene hydroperoxide	1530 ± 60 (3)[c]	1500 ± 160 (6)	425 ± 64 (3)	460 ± 49 (3)
ethyl hydroperoxide			439 ± 24 (4)	463 ± 91 (6)
hydrogen peroxide			620 ± 36 (3)	557 ± 72 (3)
DTNB reductase activity (min^{-1})[a]	1010 ± 70 (4)	1110 ± 80 (4)	ND[d]	ND
transhydrogenase activity (min^{-1})[a]	2730 ± 190 (7)	2730 ± 140 (7)	ND	ND
oxidase activity (min^{-1})[a]	101 ± 5 (4)	108 ± 4 (4)	ND	ND
ϵ_{450} of bound FAD ($M^{-1}\,cm^{-1}$)	13050 ± 310 (4)	13080 ± 180 (4)	ND	ND
A_{280}/A_{450}	5.4−9.4	5.6−6.0	ND	ND
FAD content	0.45−0.87	0.86−0.92	ND	ND

[a] AhpF activities are expressed as micromoles of NADH consumed per minute per micromole of FAD. [b] AhpF samples were assayed in the presence of 5 nmol of recombinant AhpC. AhpC samples were assayed in the presence of 5 nmol of recombinant AhpF. AhpC activities are expressed as micromoles of NADH consumed per minute per micromole of subunits. [c] Results are reported as mean ± standard error, with the number of replicates in parentheses. [d] ND = not determined.

oxidase-independent pyridine nucleotide consumption, and second, it allows the quantitative evaluation of each protein activity using different pyridine nucleotide and hydroperoxide substrates. In each assay, recombinant and natural AhpF and AhpC proteins showed essentially identical turnover numbers, at 1500 min^{-1} (26 u/mg) and 440 min^{-1} (22 u/mg), respectively, with NADH and cumene hydroperoxide (AhpC-dependent peroxidase assays have been observed to give some variation in rates on different days, ranging from about 420 to about 660 min^{-1}).

Ammonium sulfate was included in the assay mixtures on the basis of our initial observation that it surpasses bovine serum albumin, polyethylene glycol, and glycerol in enhancing catalytic activities. Subsequent studies have shown that, in all cases where the activating effect of ammonium sulfate is seen, this effect plateaus at 50 or 100 mM. Interestingly, AhpF-dependent peroxidase activity was enhanced by at least 2-fold in the presence of 100 mM ammonium sulfate, while the AhpC-dependent peroxidase activity was unaffected by ammonium sulfate.

In the assays described here, background rates in the absence of AhpC ranged from 1 to 10%. Indeed, AhpF in the absence of AhpC has a low level of peroxidatic activity, more noticeable in the AhpC-dependent peroxidase assay where it is present at 5 μM (rates of about 2.3 min^{-1} for AhpF were observed for both cumene hydroperoxide and hydrogen peroxide in the absence of AhpC). Turnover with AhpC is absolutely dependent on the inclusion of AhpF in the reaction mixture. Decreasing levels of either hydroperoxide or pyridine nucleotide substrates to 250 and 75 μM, respectively, did not significantly change the initial rates observed in either assay, indicating that substrates were at saturating levels. A comparison of three very different hydroperoxide substrates, cumene hydroperoxide, ethyl hydroperoxide, and hydrogen peroxide, showed that AhpC-dependent peroxidase activity was nearly the same for all three substrates, at 443, 453, and 589 min^{-1}, respectively, differing from an earlier report that reduction of hydrogen peroxide was not catalyzed by this enzyme system (Jacobson et al., 1989). When NADPH was used in place of NADH in AhpF-dependent hydroperoxidase assays, the activity was much lower, prompting a more detailed investigation into relative K_m values of AhpF for each pyridine nucleotide through measurement of the AhpF-catalyzed reduction of the DTNB disulfide (see below).

Comparison of NADH and NADPH as Substrates Using DTNB Reductase Assays. A part of the evidence in support of the proposed involvement of cystine disulfide reduction in the catalytic cycle of the AhpF and AhpC proteins was the demonstration that AhpF possesses DTNB reductase activity, reducing each mole of DTNB to 2 mol of TNB anion during turnover. This assay was expected to involve both FAD and cystine disulfide redox centers within AhpF and has been used here as an assay which most closely mimics the normal turnover of AhpF with AhpC plus peroxide, without the complication of having to consider protein−protein interactions in the two-protein system.

As with the AhpF-dependent peroxidase assays, DTNB reductase activities were higher (by about 2-fold) in the presence of ammonium sulfate; in contrast, higher pH Tris buffers gave enhanced rates relative to neutral pH phosphate buffers. Under either aerobic or anaerobic conditions, the optimal turnover number of AhpF with DTNB and NADH was about 1100 min^{-1}, or 19 u/mg (Table 1); this rate is comparable to that of 1500 min^{-1} for the AhpF-dependent peroxidase activity.

Kinetic studies with varied concentrations of both substrates were carried out using the readily available recombinant enzyme and a stopped flow spectrophotometer for rapid mixing and analysis. Using this assay, we were able to obtain reliable rates at concentrations of NADH as low as 0.2 μM (it should be noted that the high degree of sensitivity of this assay is partly a result of monitoring TNB production rather than NADH oxidation, taking advantage of the 2 mol of TNB generated for each mole of NADH oxidized and the higher extinction coefficient of TNB relative to NADH). AhpF (20 nM final concentration) was preincubated with DTNB (150−1000 μM) in buffer plus ammonium sulfate at 25 °C and then mixed rapidly with the appropriate concentration of pyridine nucleotide in the same ammonium sulfate-containing buffer. Double reciprocal plots of the rates obtained by varying concentrations of one substrate while holding the other constant gave parallel lines in each case. Secondary plots to extrapolate $K_{m,app}$ and $V_{max,app}$ values to infinite substrate concentrations yield a true k_{cat} of about 106 s^{-1} for AhpF and a K_m value of 2.2 μM for NADH (the K_m for DTNB is poorly defined but is probably in the low millimolar range).

In an effort to more specifically evaluate the effect of including ammonium sulfate in the assay buffer, experiments where the standard DTNB concentration of 500 μM was used and the NADH concentration was varied were also carried out in the absence or presence of other salts at an ionic strength equivalent to that of 0.1 M $(NH_4)_2SO_4$ (0.1 M K_2SO_4,

Table 2: Kinetic Analyses of the DTNB Reductase Activity of AhpF

pyridine nucleotide substrate used	[DTNB] (μM)	$V_{max,app}$ (s^{-1})[a]	$K_{m,app}$ for NAD(P)H (μM)[a]
NADH[b]	150	4.88 ± 0.11	0.30 ± 0.03
	500	14.7 ± 1.0[c]	0.58 ± 0.14[c]
	1000	28.4 ± 0.5	1.41 ± 0.08
NADPH[d]	500	10.6 ± 0.7	219 ± 39

[a] Curve fitting to the Michaelis−Menten equation was carried out using the program ENZFITTER. [b] NADH concentrations were varied from 0.2 to 8 μM. [c] Overall results were obtained using data gathered from three independent analyses. [d] NADPH concentrations were varied from 50 to 1000 μM.

0.3 M KCl, and 0.3 M NH_4Cl). Values for $V_{max,app}$ and $K_{m,app}$ for NADH in the absence of added salt were difficult to obtain given the considerable interference of substrate inhibition when NADH concentrations of 20 μM and higher are used. Nonetheless, results suggested that the addition of salt results in at least a 10-fold decrease in $K_{m,app}$ for NADH and essentially no change in the $V_{max,app}$ value. All salts tested affected these kinetic parameters in about the same way, indicating that the increased ionic strength, and not specific effects of ammonium ion itself, causes the drop in $K_{m,app}$ for NADH.

Evaluation of the $K_{m,app}$ values for NADH and NADPH at a single concentration of DTNB (500 μM) showed that NADH is a significantly better substrate for AhpF than is NADPH under these conditions (Table 2). The $K_{m,app}$ value for NADPH is quite high relative to that of NADH (219 *vs* 0.58 μM) but is still rather accurately determined as the higher concentrations of NADPH used did not result in the strong substrate inhibition seen for NADH. $V_{max,app}$ values, on the other hand, were very similar using the two pyridine nucleotide substrates.

Transhydrogenase and Oxidase Activities of AhpF. As a flavoprotein dehydrogenase, AhpF also possesses diaphorase and low oxidase activities with reduced pyridine nucleotides. Another assay which we have used with great success in analyzing FAD-dependent activity of AhpF and its derivatives and mutants has been the transhydrogenase assay, whereby net transfer of hydride ion from NADH [$E°_{red}$ (NAD^+/NADH) = −320 mV; Kaplan et al., 1956] to $AcPyAD^+$ [$E°_{red}$ ($AcPyAD^+$/AcPyADH) = −248 mV; Massey & Palmer, 1962] is monitored.

Like with peroxidase and DTNB reductase activities, AhpF-catalyzed transhydrogenase assays give higher rates in the presence of 100 mM ammonium sulfate; optimal transhydrogenase activities are obtained in Tris buffer measured under aerobic rather than anaerobic conditions [natural and recombinant AhpF proteins give a turnover number of about 2730 min^{-1} (48 u/mg) under these conditions (Table 1)].

Oxidase assays of AhpF using air-saturated buffers were the only AhpF-dependent assays unaffected by the addition of ammonium sulfate. In potassium phosphate buffer at pH 7.5, the AhpF-catalyzed turnover number for oxygen with NADH at 25 °C under standard conditions was about 100 min^{-1} (∼1.8 u/mg), considerably lower than any of the other AhpF-dependent rates measured, and both recombinant and natural AhpF proteins gave identical rates (Table 1).

Effects of Thiol Reagents on Reduced Alkyl Hydroperoxide Reductase Proteins. As described in Experimental Procedures, AhpF was preincubated under anaerobic conditions with thiol reagents in the presence or absence of NADH prior to catalytic activity assays. *N*-Ethylmaleimide was the most potent thiol reagent tested; anaerobic incubation of 25 μM inhibitor with AhpF in the presence of NADH for 15 min resulted in remaining DTNB reductase activities of <1, 46, and ∼80% for *N*-ethylmaleimide, iodoacetamide, and sodium arsenite, respectively. These results were in qualitative agreement with studies by Jacobson et al., where thiol reagents were added directly to the cumene hydroperoxide reductase assay mixture, although much higher concentrations of inhibitors were required for inhibition during turnover (Jacobson et al., 1989). Kinetic analyses of the inactivation of AhpF by varying concentrations of *N*-ethylmaleimide as assessed by aerobic DTNB reductase assays (Figure 4) showed no evidence of kinetic irreversibility, as indicated by the extrapolation of k_{obs} *vs* [*N*-ethylmaleimide] to approximately 0 at 0 inhibitor concentration (the corresponding second order rate constant was 188 M^{-1} s^{-1}). Preincubation of AhpF with 25 μM *N*-ethylmaleimide in the absence of NADH results in no measurable inactivation of AhpF. Preincubation with NADH alone is also without effect on activity.

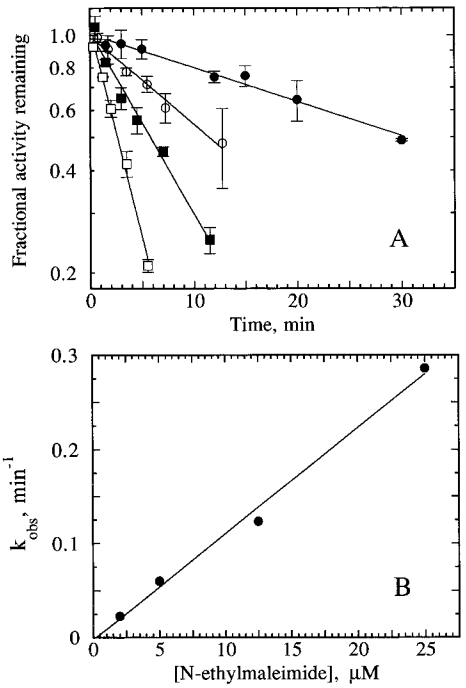

FIGURE 4: Inhibition of DTNB reductase activity by pretreatment of reduced AhpF with *N*-ethylmaleimide. Prior to aerobic assay of the NADH-dependent DTNB reductase activity of AhpF (carried out in 1 mL of an assay mixture containing 50 μM NADH, 500 μM DTNB, and 1.4−2.1 pmol of AhpF in 50 mM Tris-HCl at pH 8.0 with 100 mM ammonium sulfate and 0.5 mM EDTA), 700 nM AhpF was made anaerobic for 10 min in 25 mM potassium phosphate buffer at pH 7.0 containing 0.5 mM EDTA and then mixed with 105 μM NADH plus varying concentrations of *N*-ethylmaleimide. Shown in panel A are the data from at least two experiments per concentration, normalized to extrapolated activity values of 100%, for AhpF which was preincubated with NADH plus 2 μM (closed circles), 5 μM (open circles), 12.5 μM (closed squares), and 25 μM (open squares) *N*-ethylmaleimide. Pseudo-first order rate constants obtained at each concentration of *N*-ethylmaleimide are plotted in panel B *vs* [*N*-ethylmaleimide] and were used to obtain the second order rate constant for *N*-ethylmaleimide inactivation of reduced AhpF, 188 M^{-1} s^{-1}. Neither 105 μM NADH nor 25 μM *N*-ethylmaleimide alone preincubated anaerobically with AhpF resulted in measurable inhibition of DTNB reductase activity (data not shown).

AhpF preincubated with 25 μM *N*-ethylmaleimide under reducing conditions also loses AhpF-dependent peroxidase activity at a rate very similar to that of the loss of DTNB reductase activity (preincubation for 17 min prior to assay causes AhpF-dependent peroxidase activity to drop to 2.9%). In contrast, treatment for 20 min or more results in full retention of transhydrogenase and oxidase activities of AhpF, demonstrating the insensitivity of these enzymatic activities to thiol modification.

Similar analyses of thiol reagent effects on AhpC were carried out with preincubations performed directly in the sidearm of the anaerobic cuvette followed by AhpC-dependent peroxidase assays. Preincubation mixtures also contained a small amount of AhpF to mediate electron transfer between NADH and AhpC. Although it was recognized that rapid inactivation of reduced AhpF by the thiol reagents could interfere with the generation of reduced AhpC available for modification, mixing of AhpF and NADH with AhpC prior to addition of the thiol reagent apparently avoided this potential problem [subsequent enzymatic assay of the treated AhpC was performed by addition of a large amount (5 μM) of untreated AhpF]. As shown for AhpF, AhpC was inhibited by thiol reagents only if NADH was present during the preincubation period. Again, *N*-ethylmaleimide was a more potent inhibitor of AhpC-dependent peroxidase activity than was iodoacetamide, although sodium arsenite also showed considerable inhibition of reduced AhpC (30 min incubation of AhpC under reducing conditions with 25 μM *N*-ethylmaleimide, iodoacetamide, or sodium arsenite dropped AhpC-dependent peroxidase activities to 4, 53, and 32% of controls, respectively).

DISCUSSION

We report herein the individual overexpression and purification from *E. coli* of recombinant *S. typhimurium* alkyl hydroperoxide reductase proteins, AhpF and AhpC. Both purified proteins analyzed on SDS−polyacrylamide gels under reducing conditions give single bands corresponding to the expected size of these proteins; only AhpC migrates as a dimer under nonreducing conditions (Figure 2), indicating the presence of intersubunit disulfide bonds in AhpC, but not AhpF. Specific activities determined for AhpF and AhpC proteins from both sources have fully confirmed the identity of the recombinant proteins with those isolated directly from *Salmonella* (Table 1). Functional analyses of the recombinant proteins *in vivo* also confirmed their ability to protect against cumene hydroperoxide cytotoxicity; both proteins coexpressed confer maximal protection to the bacterial cells (Figure 3).

Assay procedures developed in our laboratory have given us a number of ways to measure specific activities for the AhpF and AhpC proteins. The peroxidase assay, requiring both proteins, is carried out anaerobically to avoid turnover of reduced AhpF with oxygen instead of AhpC during the assay (anaerobiosis is apparently unnecessary where AhpC is in great excess over AhpF; Niimura et al., 1995). The rate of NADH (or NADPH) oxidation is monitored directly at 340 nm, and specific activities for each protein are determined in the presence of an excess of the other protein. Given the reaction conditions used, the result that turnover of AhpF in the presence of excess AhpC is approximately 3-fold higher than that of AhpC in the presence of excess AhpF (1500 *vs* 440 min^{-1}, respectively) can be readily explained. In its initial stages, the AhpF-dependent activity is really only a measure of the rate of transfer of electrons between NADH and AhpC, since the latter protein is present at substrate amounts in its oxidized form. The lower rate observed in the AhpC-dependent assays indicates that electron transfer from AhpC to the peroxide substrate is rate-limiting in the overall reaction (the transfer of electrons from NADH to peroxide). AhpF by itself also catalyzes the pyridine nucleotide-dependent reduction of DTNB (a disulfide-containing substrate), molecular oxygen, and other oxidized pyridine nucleotides; ammonium sulfate has an activating effect on AhpF in all cases except the oxidase activity (the low rate of turnover with molecular oxygen may indicate a different rate-limiting step).

In addressing substrate specificities for the system, we have found that AhpC-dependent peroxidase assays using different hydroperoxide substrates give rates with H_2O_2 that are comparable to those with cumene hydroperoxide and ethyl hydroperoxide. This result differs from the original report by Jacobson et al. (1989); the pioneering work of these investigators established the wide substrate specificity of this system for organic hydroperoxides but indicated no activity of this system with H_2O_2 (lack of reactivity was attributed to inactivation of the proteins by H_2O_2 and may partly be a result of the somewhat different reaction conditions used). Earlier studies also indicated that both NADH and NADPH could act as electron donors for the reductase system. We have confirmed this result and determined, through kinetic analyses of the DTNB reductase activity of AhpF, that NADH is a considerably better reductant than NADPH (Table 2; $K_{m,app}$ is 0.58 and 220 μM for NADH and NADPH, respectively, at 500 μM DTNB; $V_{max,app}$ values are not different for the two pyridine nucleotides). This pyridine nucleotide specificity also applies when peroxidase activities are assessed in the presence of both proteins. Thus, NADPH can participate in catalysis by AhpF, but this substrate is probably only of significance when present at much higher concentrations than those required for NADH-dependent turnover.

In the comparison of the deduced amino acid sequence of AhpF with regions known in other proteins to be involved in NADH or NADPH binding, it is notable that AhpF is a sort of hybrid between the two groups. The most detailed analysis so far has been conducted by Scrutton et al. (1990), who showed that a combination of substitutions at different positions likely to be determinants of the pyridine nucleotide specificity, when introduced into *E. coli* glutathione reductase, resulted in a conversion of coenzyme specificity for the mutant proteins from NADPH to NADH. A comparison of AhpF sequences, focusing on some of these critical positions, indicates the presence of several of the residues needed for NADH interaction [e.g. the third Gly (Gly367) of the GXGXXG motif in the turn at the end of the first β-sheet and the beginning of the α-helix and the negatively charged residue (Glu385) at the end of the second β-sheet which presumably interacts with the 2′-OH of the ribose of NADH]. There is also a basic residue (Lys391) in a similar position in AhpF as one of the two arginines (Arg204) of *E. coli* glutathione reductase which interact with the 2′-phosphate of NADPH and are critical for binding. Thus, although NADH would be expected to be best accommodated within the pyridine nucleotide binding domain of AhpF, NADPH may also be accommodated in part as a result of the presence of this critical basic residue.

Enzymatic activities exhibited by *S. typhimurium* AhpF have also been reported for bacterial homologues which, like AhpF, possess the N-terminal region absent from TrR. H_2O_2-forming oxidase activities, enhanced by the addition of free FAD, were previously demonstrated for the homologues from *A. xylanus* (Niimura et al., 1993) and *S. mutans* (Higuchi et al., 1993, 1994); we have reported elsewhere (Niimura et al., 1995) that H_2O_2 is also the product of the oxidase activity of AhpF and that this activity is also stimulated by FAD. Interestingly, when an excess of AhpC is included in the incubations, twice as much NADH is consumed per molecule of oxygen and no H_2O_2 is generated (i.e. the product is H_2O from the four-electron reduction of oxygen). Diaphorase activity (NADH-dependent reduction of 2,6-dichlorophenolindophenol) has also been reported for most homologues (Higuchi et al., 1993; Niimura et al., 1993; Hisae et al., 1983; Jacobson et al., 1989). Ohnishi et al. (1994) also observed NADH-dependent DTNB reductase activity for the *A. xylanus* protein; their reported rate of 61.5 min^{-1} can be compared to our value of 275 min^{-1} for AhpF obtained under similar conditions (no ammonium sulfate added and pH 7.0). Finally, recent experiments conducted cooperatively between our laboratories have demonstrated that the *A. xylanus* flavoprotein can substitute for AhpF in catalyzing NADH-dependent hydroperoxide reduction in the presence of *S. typhimurium* AhpC (Niimura et al., 1995). These studies employed varying levels of AhpC and yielded a value of about 20 μM for the $K_{m,app}$ for AhpC using either flavoprotein.

Our analyses of the sensitivities of oxidized and reduced forms of AhpF and AhpC toward inactivation by thiol reagents have provided strong support for the hypothesis that redox-active disulfide centers present in both proteins are important in catalysis. Neither oxidized protein is inactivated by preincubation with *N*-ethylmaleimide, iodoacetamide, or sodium arsenite. Reduced proteins, on the other hand, are rapidly inactivated by these agents at micromolar concentrations, with *N*-ethylmaleimide being the most potent inhibitor. This result is consistent with the hypothesis that AhpF, like TrR, acts as a disulfide reductase and that nascent dithiols generated with AhpC are directly responsible for the reduction of hydroperoxide substrates. This result also demonstrates that free thiol groups of catalytic importance are accessible in the reduced forms of each protein. Our results have led us to explore the redox properties of cysteinyl residues in both proteins in further investigations, reported in the accompanying paper, to establish the mechanism for enzymatic catalysis employed by each alkyl hydroperoxide reductase protein.

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