Requirement for the Two AhpF Cystine Disulfide Centers in Catalysis of Peroxide Reduction by Alkyl Hydroperoxide Reductase†

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ABSTRACT: AhpF, the alkyl hydroperoxide reductase component which transfers electrons from pyridine nucleotides to the peroxidase protein, AhpC, possesses two redox-active disulfide centers in addition to one FAD per subunit; the primary goal of these studies has been to test for the requirement of one or both of these disulfide centers in catalysis. Two half-cystine residues of one center ($Cys_{345}Cys_{348}$) align with those of the homologous *Escherichia coli* thioredoxin reductase (TrR) sequence ($Cys_{135}Cys_{138}$), while the other two ($Cys_{129}Cys_{132}$) reside in the additional N-terminal region of AhpF which has no counterpart in TrR. We have employed site-directed mutagenesis techniques to generate four mutants of AhpF, including one which removes the N-terminal disulfide ($Ser_{129}Ser_{132}$) and three which perturb the TrR-like disulfide center ($Ser_{345}Ser_{348}$, $Ser_{345}Cys_{348}$, and $Cys_{345}Ser_{348}$). Fluorescence, absorbance, and circular dichroism spectra show relatively small perturbations for mutations at the disulfide center proximal to the flavin ($Cys_{345}Cys_{348}$) and no changes for the $Ser_{129}Ser_{132}$ mutant; identical circular dichroism spectra in the ultraviolet region indicate unchanged secondary structures in all mutants studied. Oxidase and transhydrogenase activities are preserved in all mutants, indicating no role for cystine redox centers in these activities. Both DTNB and AhpC reduction by AhpF are dramatically affected by each of these mutations, dropping to less than 5% for DTNB reductase activity and to less than 2% for peroxidase activity in the presence of AhpC. Reductive titrations confirm the absence of one redox center in each mutant; even in the absence of $Cys_{345}Cys_{348}$, the N-terminal redox center can be reduced, although only slowly. These results emphasize the necessity for both redox-active disulfide centers in AhpF for catalysis of disulfide reductase activity and support a direct role for $Cys_{129}Cys_{132}$ in mediating electron transfer between $Cys_{345}Cys_{348}$ and the AhpC active-site disulfide.

The alkyl hydroperoxide reductase (AhpR)[1] enzyme system from *Salmonella typhimurium* is composed of two proteins, AhpF and AhpC, which together catalyze the NAD(P)H-dependent reduction of organic hydroperoxides and hydrogen peroxide. Deletion mutants lacking this system (*ahp*$^-$) or the transcriptional regulator of these and other oxidative stress-linked proteins (*oxyR*$^-$) are hypersensitive toward hydrogen peroxide or cumene hydroperoxide treatment and, in the latter case, accumulate more mutations during aerobic growth (*1*−*3*). Expression of recombinant AhpR proteins in *oxyR*$^-$ strains lowers the spontaneous mutagenesis levels to those of wild-type *oxyR* strains, clearly implicating these proteins as critical components in cellular defense systems which protect against oxidative damage to DNA (*1*, *2*). The cytotoxicity of cumene hydroperoxide is also substantially decreased in strains overexpressing both AhpR proteins; either protein independently overexpressed in *Escherichia coli* is partially protective (*4*).

Characterization of the purified AhpF and AhpC proteins has clarified the role of each in catalysis of peroxide reduction. AhpF, a flavoprotein with two redox-active disulfide centers per subunit ($Cys_{129}Cys_{132}$ and $Cys_{345}Cys_{348}$), catalyzes electron transfer between reduced pyridine nucleotides and acceptor substrates such as oxygen, DTNB, and AhpC (*4*−*6*). Reduced AhpC, which contains two free thiol groups per subunit, is a direct reductant of organic hydroperoxides and hydrogen peroxide; oxidation of AhpC returns the protein to its native form with two intersubunit disulfide bonds per dimer (*4*, *6*). Recent investigations have demonstrated that the disulfide bonds of AhpC are formed between Cys_{46} and Cys_{165} on the other subunit, implying the presence of two identical active-site cystine centers per AhpC dimer (*7*). That catalytic cysteine residues present in each reduced protein are required for catalysis of peroxide reduction has been demonstrated using thiol-modifying agents (*4*). On the basis of these findings and work with other flavoprotein systems, electron transfer is expected to occur from NAD(P)H to FAD to one or both redox-active disulfide center(s) of AhpF, from AhpF to AhpC through thiol−disulfide interchange, and from a nascent catalytic thiol of AhpC to ROOH (Scheme 1). Through analogy with other non-heme peroxidase systems (*8*, *9*) and recent experimental data (*7*), this last step probably involves transient formation of a cysteine sulfenic acid (Cys-SOH), followed by condensa-

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[1] Abbreviations: AhpR, alkyl hydroperoxide reductase; TrR, thioredoxin reductase; Tr, thioredoxin; $AcPyAD^+$, oxidized 3-acetylpyridine adenine dinucleotide; AcPyADH, reduced 3-acetylpyridine adenine dinucleotide; AAD^+, 3-aminopyridine adenine dinucleotide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); TNB, 2-nitro-5-thiobenzoate; NTSB, 2-nitro-5-thiosulfobenzoate; SDS, sodium dodecyl sulfate.

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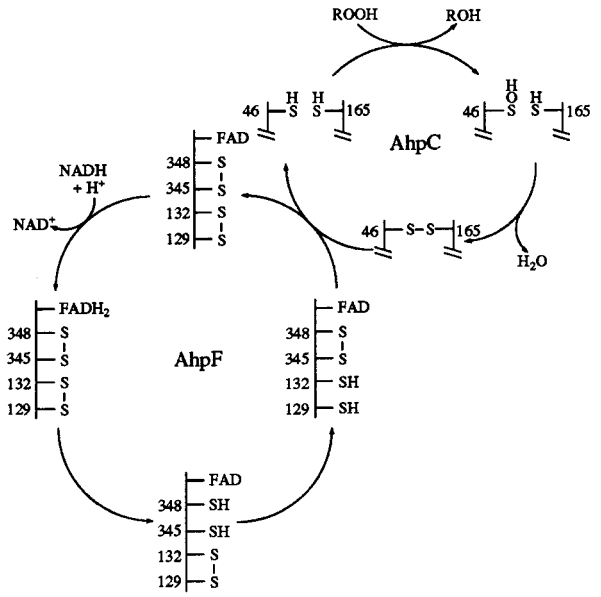
Scheme 1



tion of this species with the other active-site cysteine to reform the disulfide bond.

Information pertaining to AhpF structure can be derived from comparisons of its coding sequence (*10*) with that of thioredoxin reductase from *Escherichia coli* (TrR; *11*), a homologue bearing 35% identity at the amino acid level with the C-terminal 60% of AhpF (*12*; Figure 1). Pyridine nucleotide and FAD binding motifs, as well as the well-characterized redox-active disulfide center of TrR, are all present in AhpF; the two additional cysteinyl residues encoded within the C-terminal region of the AhpF structural gene that are not present in TrR (Cys_{476} and Cys_{489}) play no structural or catalytic role in AhpF as assessed by site-directed mutagenesis (*6*). Modeling of this portion of AhpF based on the known TrR structure (*13*) has suggested that the C-terminus of AhpF can adopt a structure quite similar to that of TrR, with Cys_{345} and Cys_{348} in close proximity to the isoalloxazine ring of the bound FAD (*14*). The C-terminal and N-terminal portions of AhpF represent separately-folding domains linked by a proteolytically-sensitive "hinge" region as demonstrated by limited tryptic digestions (*6*; Figure 1) and independent expression of the two fragments through mutagenesis techniques (*14*).

Like the wide variety of flavin-containing pyridine nucleotide:disulfide oxidoreductases characterized to date (*15*), the unique architecture of AhpF appears to impart its specialized AhpC-reducing activity. Without the N-terminal domain, the C-terminal tryptic fragment of AhpF does not catalyze peroxide reduction in the presence of AhpC (*6*); in static titrations, truncated AhpF missing the N-terminal 207 amino acids is virtually incapable of reducing AhpC (*14*). We have therefore hypothesized that the N-terminal domain of AhpF acts as an appended substrate for the TrR-like C-terminal portion of the protein, mediating electron transfer between the TrR-like $Cys_{345}Cys_{348}$ disulfide center and the redox-active disulfide of AhpC. The necessity for multiple alternating interactions between AhpF redox centers and the mounting evidence from studies of the related TrR protein (*16*) have also led us to hypothesize conformational changes within AhpF during catalysis (*6*). Studies described herein address the function of both redox-active disulfide centers of AhpF through site-directed mutagenesis approaches.

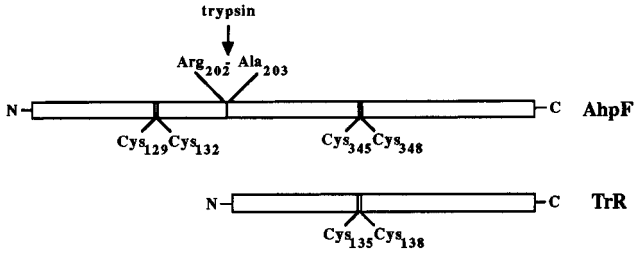


FIGURE 1: Location of the tryptic cleavage site and redox-active half-cystine residues within the AhpF protein sequence in comparison with those of thioredoxin reductase (TrR). The remaining catalytically inactive Cys_{476} and Cys_{489} of AhpF and Cys_{105} and Cys_{303} of TrR are not shown.

Structural and catalytic effects of substituting each pair of cysteine residues ($Cys_{129}Cys_{132}$, or $Cys_{345}Cys_{348}$), or Cys_{345} or Cys_{348} individually, with serine residues have been assessed in these studies. We present data that clearly establish the importance of the N-terminal disulfide center in the catalysis of AhpC (and therefore peroxide) reduction.

MATERIALS AND METHODS

Materials. NADH was purchased from Boehringer Mannheim. Sigma was the supplier of FAD, 3-acetylpyridine adenine dinucleotide ($AcPyAD^+$), 3-aminopyridine adenine dinucleotide (AAD^+), cumene hydroperoxide (80%), molecular biology grade ammonium sulfate, methyl viologen, protocatechuic acid, and protocatechuate 3,4-dioxygenase. Difco bacteriological media, sodium dithionite, and organic solvents were from Fisher. Other reagents and buffer components including DTNB, SDS, and ultrapure urea were purchased from Research Organics, Inc. Guanidine hydrochloride was from Gibco BRL. Restriction enzymes, T4 DNA ligase, calf-intestinal alkaline phosphatase, and other DNA-modifying enzymes were obtained from New England Biolabs.

[α-^{35}S]dATP was from New England Nuclear. The standard buffer used was 25 mM potassium phosphate at pH 7.0, with 1 mM EDTA, unless otherwise mentioned.

Mutagenesis, Expression, and Purification of the Mutant Enzymes. In order to replace Cys_{345} and Cys_{348} or Cys_{129} and Cys_{132} of AhpF with serine or alanine residues for the generation of the double mutants, the following mutagenic oligonucleotides were designed: 5′-GTCACCTATT/GCCCCGCACT/GCCGACGGTCCG-3′ and 5′-TTCACTCTCCT/GCCCATAACT/GCCCCGG-3′ (underlined nucleotides are the sites of the new codons; T and G are in equal amounts in the first position of these codons). The oligonucleotides synthesized for individual replacement of Cys_{345} and Cys_{348}, respectively, were 5′-GTCACCTATT/GCCCCGCACTGCGACGGTCCG-3′ and 5′-GTCACCTATTGTCCGCACT/GCCGACGGTCCG-3′ (oligonucleotide synthesis was performed in the DNA Synthesis Core Laboratory of the Comprehensive Cancer Center of Wake Forest University, supported in part by NIH Grant CA 12197). To generate the template for mutagenesis at codons 129 and 132, the 526 bp *Eco*RI−*Eag*I restriction fragment bearing the coding region for a portion of AhpF was excised from the expression vector, pAF1 (*2*), and ligated into the corresponding restriction sites of pBluescriptII SK(+) from Stratagene using standard DNA manipulation techniques (*17*); this new plasmid was designated pBlF1 (Figure 2). All other mutants were created using template from the same phagemid vector bearing the entire coding region for AhpF (pBlF2; *4*; Figure

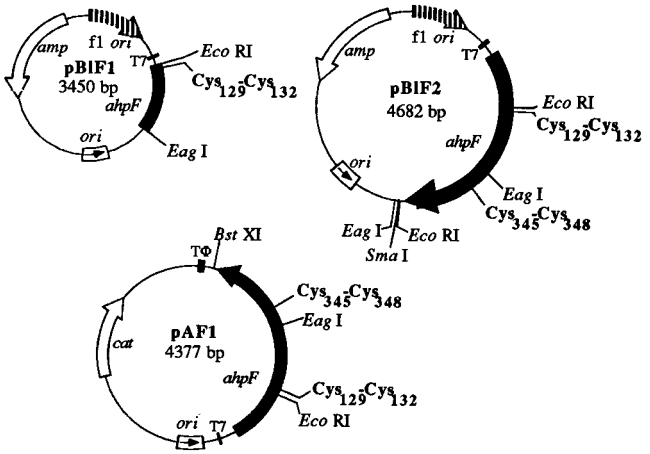


FIGURE 2: Recombinant plasmids used in mutagenesis and expression of AhpF mutants. Plasmids were constructed as described under Materials and Methods using the indicated restriction sites. TΦ refers to the T7 termination sequence from pET-11, T7 to the T7 promoter sequence, *cat* to the chloramphenicol acetyltransferase gene, and *amp* to the β-lactamase gene conferring ampicillin resistance.

2). Single-stranded DNA was prepared using single-stranded rescue by R408 helper phage from Stratagene (*18*). Following mutagenesis with the Sculptor *in vitro* mutagenesis kit from Amersham, mutants were screened and appropriate mutations identified by sequencing of single-stranded DNA using the Sequenase Version 2 sequencing kit from United States Biochemicals; only the serine mutants were chosen for subcloning into expression vectors at this stage. To create the expression plasmid for $Ser_{129}Ser_{132}$[2] AhpF, the *Eco*RI−*Eag*I fragment containing the mutated region was subcloned back into the identical sites of pAF1 following dephosphorylation of the digested vector fragment. Expression plasmids for $Ser_{345}Cys_{348}$, $Cys_{345}Ser_{348}$, and $Ser_{345}Ser_{348}$ were similarly created by transfer of the 734 bp *Eag*I−*Sma*I fragment from the mutated phagemid to the *Eag*I and blunted *Bst*XI sites of pAF1. Confirmation that the correct mutations were present was again obtained by DNA sequence analysis of the entire new restriction fragments within the expression vectors. Recombinant AhpF mutant proteins were expressed and purified as previously reported for the wild-type recombinant enzyme (*4*).

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 essentially all enzymatic assay and anaerobic titration experiments. Several activity assays were also performed on a thermostatted Applied Photophysics DX.17MV stopped-flow spectrofluorometer. Circular dichroism and fluorescence spectra were recorded using a Jasco J-720 spectropolarimeter and an SLM Aminco Bowman Series 2 luminescence spectrophotometer, respectively. Fluorescence intensity measurements were compared each time with that of a 8.8 μM FAD standard solution ($A_{450} = 0.1$); the concentrations of wild-type and mutant proteins ranged between 6.9 and 7.6 μM, corresponding to an A_{450} of $\sim$0.09. Extinction coefficients used for all reduced and oxidized pyridine nucleotides, free FAD, and TNB were those previously reported (*4*). The molar extinction coefficients of the protein-bound FAD at 445 or 449 nm were determined by release of the flavin cofactor with 4 M guanidine hydrochloride and quantitation of the corresponding free FAD (*4*).

Anaerobic titrations were carried out essentially as described previously (*6*). Dithionite and NADH titrating solutions were prepared in buffer bubbled for 20−30 min with oxygen-free nitrogen, loaded into the titrating syringe, and standardized prior to each experiment. An oxygen-scrubbing system consisting of protocatechuate 3,4-dioxygenase and protocatechuic acid was included in all NADH titration experiments, and methyl viologen was added at a molar ratio of 1:100 relative to the AhpF protein to promote equilibration between redox centers during dithionite titrations (*6*). Spectral data were not corrected for dilution, which was <2.5% overall.

Activity and Thiol Assays. Anaerobic AhpF-dependent peroxidase assays with cumene hydroperoxide were carried out essentially as described previously (*4*), but employed a stopped-flow spectrophotometer and concentrations of 20 μM AhpC and 10 μM mutant AhpF proteins (or 0.1 μM wild-type AhpF). DTNB reductase assays were performed as described previously (*4*) with the exceptions that NADH was added at 200 μM and plastic or glass cuvettes were used to prevent nonenzymatic decomposition of DTNB by ultraviolet light in the diode array spectrophotometer. Transhydrogenase and oxidase assays followed previously described protocols (*4*).

Assays for free thiol groups were carried out either by incubation of protein with nitrogen-bubbled guanidine hydrochloride at a final concentration of 4 M for several minutes followed by addition of 100 μM DTNB, or by similar additions of 6 M guanidine hydrochloride and 100 μM NTSB, a sulfite-insensitive reagent described by Thannhauser et al. (*19*) which can be used following dithionite titrations. All thiol assays were performed in 0.5−1.0 mL volumes containing sufficient protein to generate A_{412} values between 0.5 and 1.0.

RESULTS

Spectral Analyses of AhpF Mutants. Comparisons of the visible absorbance spectra of $Ser_{129}Ser_{132}$, $Ser_{345}Ser_{348}$, and $Ser_{345}Cys_{348}$ mutant AhpF proteins, as summarized in Table 1, indicated virtually identical spectral properties for these mutants compared with the wild-type enzyme. In contrast, the visible spectrum of the $Cys_{345}Ser_{348}$ mutant of AhpF exhibits markedly blue-shifted flavin peaks, with maxima at 373 and 445 nm (compared with 381 and 449 nm for wild-type AhpF), and a significantly lowered extinction coefficient for the 373 nm absorbance band (Figure 3). Extinction coefficients for all four mutants at 449 or 445 nm are similar to, but slightly less than, the value for the wild-type flavoprotein. Unlike spectral characteristics of a comparable mutant of TrR, $Ser_{135}Cys_{138}$ (*20*), no absorbance band beyond 500 nm which would correspond to a thiolate → FAD charge-transfer interaction is exhibited by the $Ser_{345}Cys_{348}$ mutant of AhpF, even when shifted to conditions of higher pH (up to pH 8.5) or high ammonium chloride concentration (up to 0.5 M).

Circular dichroism spectra of the four mutants are essentially identical with that of wild-type AhpF in the far-ultraviolet region, a strong indication that none of the

[2] Mutants studied herein are designated $Ser_{129}Ser_{132}$, $Ser_{345}Ser_{348}$, $Ser_{345}Cys_{348}$, and $Cys_{345}Ser_{348}$ using nomenclature as in Miller et al. (*23*). Any of the six cysteine residues not included in each designation have not been altered by mutagenesis.

Table 1: Spectral Properties of Wild-Type and Mutant AhpF Proteins

	wild type	$Ser_{129}Ser_{132}$	$Ser_{345}Ser_{348}$	$Ser_{345}Cys_{348}$	$Cys_{345}Ser_{348}$
molar extinction coefficient[a]	13 000 ± 660	12 400 ± 300	11 800 ± 600	12 500 ± 900	12 300 ± 800
λ_{max} of two flavin peaks (nm)	381, 449	381, 449	378, 449	381, 449	373, 445
ratio of absorbances[b]	1.04	1.03	1.07	1.00	1.20
fluorescence[c]	1.00	1.00	1.32	1.00	0.54

[a] Results are for 449 nm or, in the case of $Cys_{345}Ser_{348}$, for 445 nm, and are shown as mean ± standard deviation (four replicates) expressed as M^{-1} cm^{-1}. [b] The ratio is of the absorbance of the flavin peak at 445/449 nm divided by that of the flavin peak at 373/381 nm. [c] Fluorescence intensity is shown relative to wild-type AhpF at 1.0, a value which is 24% that of an equivalent concentration of free FAD.

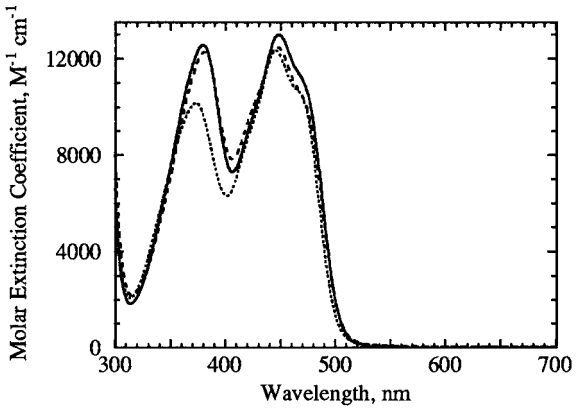


FIGURE 3: Comparison of wild-type, $Ser_{345}Cys_{348}$, and $Cys_{345}Ser_{348}$ AhpF visible absorbance spectra. The proteins were in standard phosphate buffer at neutral pH and room temperature; spectra shown are for wild-type (solid line), $Cys_{345}Ser_{348}$ (dashed line), and $Ser_{345}Cys_{348}$ (dotted line) proteins.

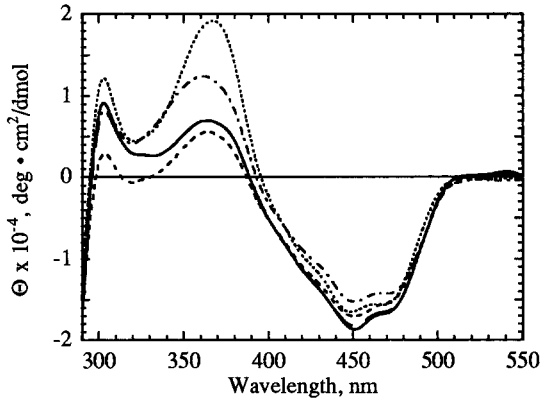


FIGURE 4: Comparison of visible circular dichroism spectra for wild-type and mutant AhpF proteins. Spectra of wild-type and mutant proteins were obtained for 61−68 μM of each protein in standard phosphate buffer at pH 7 and room temperature. Measurements were taken in 0.5 nm increments from 550 to 290 nm in a 1 cm path length cuvette. Each spectrum is the average of eight scans; smoothing of the data was performed using the default parameters within the Jasco J-720 software. Spectra shown are for wild-type (solid line), $Ser_{129}Ser_{132}$ (long dashed line, almost completely overlapping with the wild-type spectrum), $Ser_{345}Ser_{348}$ (short dashed line), $Ser_{345}Cys_{348}$ (dashed−dotted line), and $Cys_{345}Ser_{348}$ (dotted line) AhpF proteins.

mutations is accompanied by major changes in the secondary structures of the proteins (data not shown). Interestingly, visible circular dichroism spectra shown in Figure 4 are perturbed for all mutants in which the mutation(s) is (are) in the disulfide center that is expected to be proximal to the flavin ($Ser_{345}Cys_{348}$, $Cys_{345}Ser_{348}$, and $Ser_{345}Ser_{348}$); the spectrum of the $Ser_{129}Ser_{132}$ mutant is identical with that of wild-type AhpF. Wild-type, $Ser_{129}Ser_{132}$, and $Ser_{345}Cys_{348}$ mutant proteins exhibited a similar fluorescence intensity, whereas the emission intensity of the $Ser_{345}Ser_{348}$ mutant protein was increased by 32% and that of the $Cys_{345}Ser_{348}$ protein was decreased by 46% relative to the wild-type enzyme.

Flavin-Dependent Transhydrogenase and Oxidase Activities of AhpF Mutants. Two flavin-mediated activities catalyzed by AhpF, transhydrogenase activity which measures hydride transfer from NADH to a pyridine nucleotide analogue of higher potential ($AcPyAD^+$), and oxidase activity which arises from the transfer of two electrons from NADH to O_2, have been very useful in comparing mutant and truncated AhpF proteins as an assessment of their proper folding and appropriate flavin binding. Previous results had indicated that neither of these activities were sensitive to inactivation by thiol reagents (*4*). Removal of 202 amino acids from the N-terminus of AhpF by limited proteolysis was also without effect on these activities (*6*). Our results in this study, summarized in Table 2, indicate that replacement of the N-terminal disulfide center with serine residues has no effect on either activity. Oxidase activity is also unchanged for $Cys_{345}Ser_{348}$ and $Ser_{345}Ser_{348}$ mutants, while the $Ser_{345}Cys_{348}$ mutant exhibits oxidase activity at nearly twice the level of the other mutants and wild-type AhpF. Transhydrogenase activities of $Ser_{345}Cys_{348}$, $Cys_{345}Ser_{348}$, and $Ser_{345}Ser_{348}$ span a range of values, from 80 to 230% relative to the wild-type and $Ser_{129}Ser_{132}$ proteins. It appears that the absence of a cysteine at position 345 imparts higher transhydrogenase activity, while the presence of Cys_{345} in the absence of Cys_{348} decreases the level of activity. It is clear, in any case, that neither disulfide center of AhpF is required for efficient electron transfer between pyridine nucleotides.

Effects of Cysteine Mutations on NADH-Dependent DTNB and AhpC/Peroxide Reductase Activities of AhpF. Efficient catalytic reduction of the disulfide bond of DTNB to release the chromophoric TNB anion was demonstrated previously for the wild-type AhpF protein (*4*, *5*). Substitution by serine of one or more of the AhpF cysteine residues under investigation in this study causes serious impairment of this activity (Table 2). The mutant missing the N-terminal disulfide center, $Ser_{129}Ser_{132}$, exhibits about 5% of the activity of the wild-type enzyme, while mutations of cysteinyl residues within the C-terminal disulfide center also result in a drop in activity to 2−5% of the wild-type catalytic rate. Both intact disulfide centers are thus required for efficient electron transfer to DTNB.

AhpF-dependent peroxidase assays carried out anaerobically in the presence of saturating substrates (300 μM NADH and 1 mM cumene hydroperoxide) and an excess of AhpC (20 μM) indicated very low AhpC-mediated peroxidase activity (<2%) for all four mutant AhpF proteins (Table 2). Efforts to use SDS−polyacrylamide gels of iodoacetamide-treated proteins to demonstrate AhpC reduction (detected as conversion of the covalent dimer to monomer; *4*) within 30 s after anaerobic addition of this protein to stoichiometric amounts of each NADH-reduced double mutant, $Ser_{129}Ser_{132}$

Table 2: Enzymatic Activities of Wild-Type and Mutant AhpF Proteins[a]

	wild type	$Ser_{129}Ser_{132}$	$Ser_{345}Ser_{348}$	$Ser_{345}Cys_{348}$	$Cys_{345}Ser_{348}$
oxidase activity[b]	196 ± 15	206 ± 10	220 ± 21	372 ± 60	234 ± 19
transhydrogenase activity[c]	1970 ± 230	2090 ± 260	4490 ± 610	4130 ± 210	1620 ± 160
peroxidase activity[d]	8050 ± 226	63.6 ± 6.6	57.0 ± 7.8	140.0 ± 7.2	69.6 ± 5.4
DTNB reductase activity[e]	810 ± 130	39 ± 3	16 ± 3	34 ± 5	20 ± 3

[a] Activity results reported as mean ± standard error are expressed as micromoles of substrate reduced per minute relative to micromoles of FAD determined at 450 nm; assay volumes were 1 mL (except in peroxidase assays using the stopped-flow spectrophotometer). [b] Results were obtained for 30 and 60 pmol of each protein and four replicates. [c] Results were obtained using 10 and 20 pmol of each protein with five replicates. [d] AhpF-dependent peroxidase activity was measured anaerobically on a stopped-flow spectrophotometer with 0.1 μM (wild type) or 10 μM (mutant) AhpF protein and 20 μM AhpC. Activities were corrected for the very low peroxidase activity in the absence of AhpC and were repeated at least 3 times. [e] DTNB reductase activities from at least four separate experiments were measured with 3−20 pmol of wild-type and 50−200 pmol of mutant AhpF proteins.

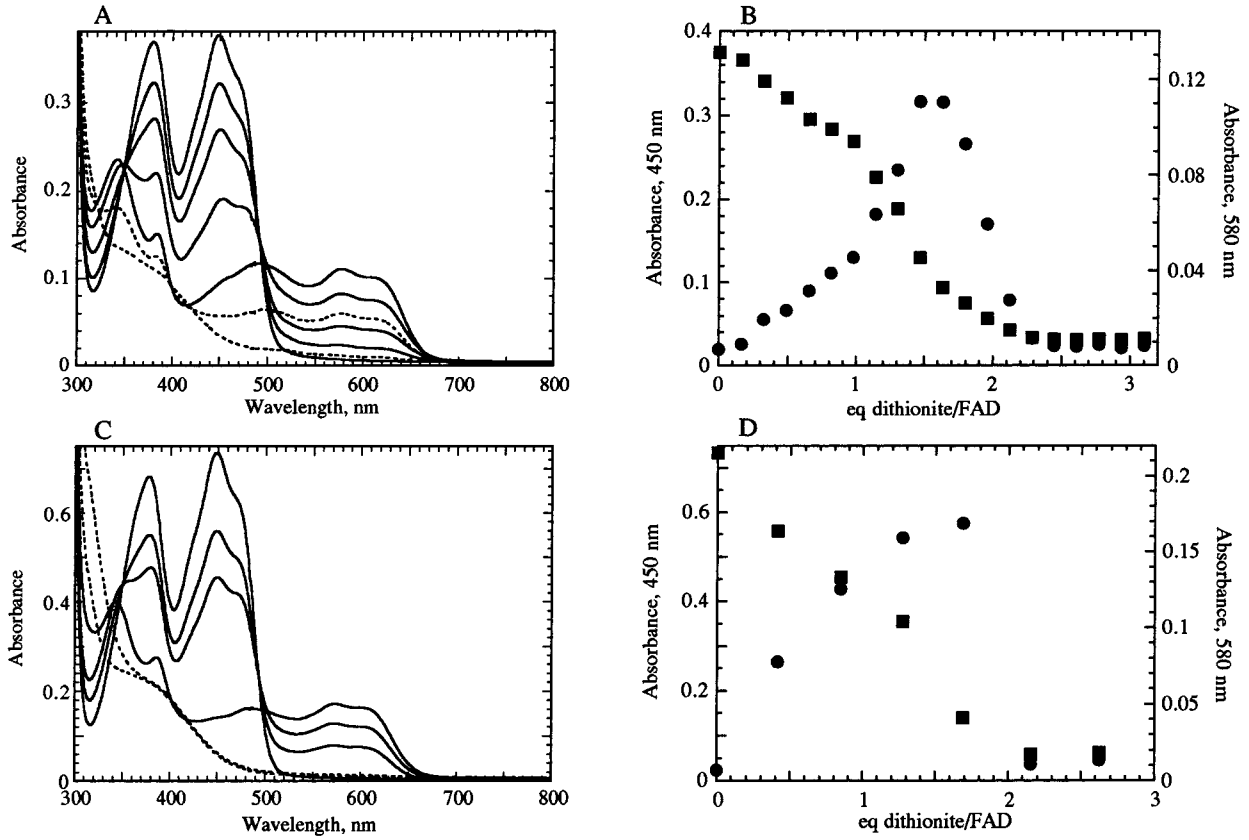


FIGURE 5: Anaerobic dithionite titrations of $Ser_{129}Ser_{132}$ and $Ser_{345}Ser_{348}$ AhpF mutants. Titrations were carried out in standard phosphate buffer at neutral pH in a total volume of 550 μL at 25 °C. $Ser_{129}Ser_{132}$ (16.4 nmol; panels A and B) and $Ser_{345}Ser_{348}$ (34.0 nmol; panels C and D) mutant proteins were titrated in the presence of methyl viologen with 1.9 and 2.9 mM anaerobic solutions of dithionite, respectively. Spectra (panels A and C) were recorded after each addition when no further absorbance changes occurred. Solid lines in panel A represent spectra obtained after the addition of 0, 0.49, 0.98, 1.31, and 1.63 equiv of dithionite/FAD in order of decreasing A_{450} and increasing A_{580}. Dashed lines in panel A indicate spectra taken after the addition of 1.96 and 2.28 equiv of dithionite/FAD in order of decreasing A_{450} and A_{580}. Similarly, solid lines in panel B represent spectra obtained after the addition of 0, 0.41, 0.84, and 1.68 equiv of dithionite/FAD, and dashed lines indicate spectra taken after the addition of 2.16 and 2.65 equiv of dithionite/FAD in order of increasing A_{320}. Panels B and D show the absorbance changes at 450 (closed squares) and 580 nm (closed circles) *versus* equiv of dithionite/FAD added.

or $Ser_{345}Ser_{348}$, of AhpF failed to detect any rapid reduction of AhpC by either mutant (data not shown). Clearly, the integrity of both disulfide centers of AhpF is obligatory for efficient transfer of electrons from AhpF to AhpC and normal catalytic reduction of hydroperoxides by the system.

Reductive Titrations of AhpF Mutants. Reductive titrations of all four AhpF mutants of interest herein give spectral changes that are qualitatively similar to those observed during NADH and dithionite titrations of wild-type AhpF (*6*). Addition of either reductant leads to spectral changes indicative of the progressive reduction of FAD and formation of significant amounts of the blue, neutral semiquinone form of the flavin (Figures 5 and 6). Further additions of dithionite lead to full reduction of the flavin; NADH, on the other hand, is a relatively poor reductant of the flavin semiquinone and does not give complete reduction of the flavin even when added in large excess. Formation of a long-wavelength charge-transfer band accounted for by $FADH_2 \rightarrow NAD^+$ interaction also occurs during NADH, but not dithionite, titrations, although the extent of formation of this species is different for some of the mutants (see below).

In all titrations, disruption of either of the two disulfide centers results in the consumption of 1 equiv less of the reductant compared with similar titrations of wild-type AhpF. As shown in Figure 5, dithionite titrations require about 2.2 equiv/FAD of the reductant to achieve complete reduction of mutant proteins, relative to ~3.2 equiv for wild-type AhpF (*6*; data not shown for $Ser_{345}Cys_{348}$ and $Cys_{345}Ser_{348}$). The apparent preferential reduction of the disulfide center relative to the flavin demonstrated by the pattern of absorbance

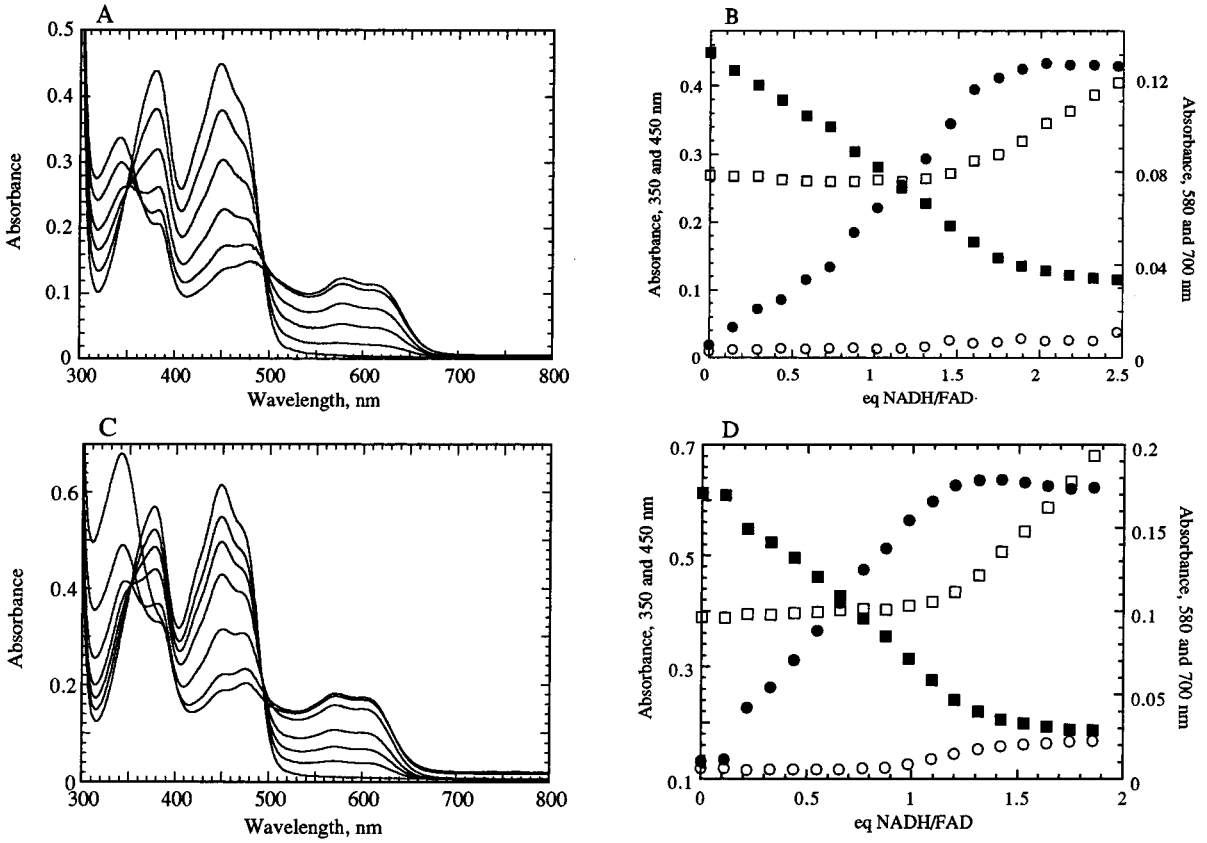


FIGURE 6: Anaerobic NADH titrations of $Ser_{129}Ser_{132}$ and $Ser_{345}Ser_{348}$ AhpF mutants. Titrations were carried out in standard phosphate buffer at neutral pH in a total volume of 550 μL at 25 °C. $Ser_{129}Ser_{132}$ (18.0 nmol; panels A and B) and $Ser_{345}Ser_{348}$ (25.0 nmol; panels C and D) mutant proteins were titrated with 1.9 and 2.5 mM anaerobic solutions of NADH, respectively. Spectra (panels A and C) were recorded after each addition when no further absorbance changes occurred. Spectra shown in panel A, in order of decreasing A_{450} and increasing A_{580}, were obtained after the addition of 0, 0.44, 0.87, 1.31, 1.60, and 1.89 equiv of NADH/FAD; similarly, spectra shown in panel C were taken after the addition of 0, 0.22, 0.44, 0.66, 0.98, 1.31, and 1.75 equiv of NADH/FAD. Panels B and D show the absorbance changes at 350 (open squares), 450 (closed squares), 580 (closed circles), and 700 nm (open circles) *versus* equiv of NADH/FAD added.

changes for the $Ser_{129}Ser_{132}$ mutant is much like that seen for the wild-type protein with two disulfide centers (*6*). Fewer points were obtained for the dithionite titration of $Ser_{345}Ser_{348}$ due to the excessive time (~12 h) required for equilibration after addition of reductant, even in the presence of methyl viologen.

During anaerobic NADH titrations, oxidation of NADH, monitored at 350 nm where the conversion of oxidized flavin to semiquinone is isosbestic, accounts for 1.3−1.5 equiv/FAD, rather than ~2.6 equiv as measured for wild-type AhpF (*6*), at the breakpoint in titrations of all mutant AhpF proteins (Figure 6; again, data not shown for $Ser_{345}Cys_{348}$ and $Cys_{345}Ser_{348}$). The extent of formation of semiquinone during these NADH titrations is similar to that seen for wild-type AhpF, at roughly 80% of total flavin by the end of the titration, although the rise in 580 nm is more linear with respect to added NADH for the $Ser_{345}Ser_{348}$ mutant (Figure 5D) than for the $Ser_{129}Ser_{132}$ mutant (Figure 5B). A quantitative indication of this difference results from a comparison of semiquinone formation in the two double mutants on addition of 0.5 equiv of NADH: 17% of the flavin has been converted to semiquinone at this stage in the titration of $Ser_{129}Ser_{132}$, while 36% of the flavin is present as semiquinone at the same point in the $Ser_{345}Ser_{348}$ titration. Preferential reduction of the disulfide center relative to the flavin is also seen for the $Ser_{129}Ser_{132}$ mutant during these titrations. Spectral changes during NADH titrations of the $Ser_{345}Ser_{348}$ mutant also take a longer time to reach equilibrium than do those with the $Ser_{129}Ser_{132}$ mutant (~20−30 min per addition relative to 10−15 min), although this rate is considerably greater than for the dithionite titration.

One notable difference between the double mutants of AhpF is the extent of formation of the long-wavelength absorbance band at 700 nm during NADH titrations. The extent of formation of this species in the $Ser_{345}Ser_{348}$ mutant is very similar to that seen during NADH titration of wild-type AhpF (*6*). The spectrum of NADH-titrated $Ser_{129}Ser_{132}$ is almost totally lacking this absorbance, much like the result seen for the truncated protein missing the entire N-terminus through residue 207 (*14*). The presence of the N-terminal disulfide center clearly has a major effect on this $FADH_2 \rightarrow NAD^+$ charge-transfer interaction. We have hypothesized either that the oxidized pyridine nucleotide is not bound as tightly by the reduced $Ser_{129}Ser_{132}$ protein, or that the predominant position and orientation of the nicotinamide ring of bound NAD^+ are not favorable for interaction with the isoalloxazine ring in this mutant. Efforts to detect differences in the binding of AAD^+ to each reduced AhpF double mutant were not, however, successful (data not shown). We are currently seeking evidence for different stable conformations of the two mutants that would support our second proposal.

That electron transfer from the reduced flavin to the N-terminal disulfide center occurs at all in the absence of Cys_{345} and Cys_{348} is surprising. This result is distinctly different from that of Ohnishi et al. (*21*) with the $Ser_{337}Ser_{340}$ (C337,340S) mutant of the *A. xylanus* AhpF homologue; only the bound FAD of this mutant is reducible by NADH or dithionite. This lack of electron transfer to the remaining

disulfide center of the $Ser_{337}Ser_{340}$ mutant of the *A. xylanus* flavoprotein was also confirmed by the unchanged thiol content of the protein at the end of the titration; our four AhpF mutants all exhibited two new thiol groups per FAD following NADH or dithionite titrations.

DISCUSSION

Studies described herein demonstrate clearly that both redox-active disulfide centers, $Cys_{129}Cys_{132}$ and $Cys_{345}Cys_{348}$, are required for catalysis of AhpC reduction by AhpF. Static titrations with NADH and dithionite reported previously (*6*) had originally established the presence of these two redox centers in AhpF in addition to the tightly bound FAD. Amino acid sequence comparisons with AhpF homologues from both Gram-negative and Gram-positive bacteria have also demonstrated absolute conservation of all four cysteinyl residues, as well as a high degree of conservation among the amino acids surrounding each center, further supporting an essential role for these residues (*6*). That the $Cys_{345}Cys_{348}$ center is in close communication with the isoalloxazine ring of the flavin is strongly suggested by the alignment of the C-terminal region of AhpF with full-length TrR from *E. coli* and by modeling based on this homology (*12*, *14*). Our spectral data confirm the proximity of $Cys_{345}Cys_{348}$ and FAD; only mutations involving this disulfide center, and not those at the $Cys_{129}Cys_{132}$ center, affect absorbance, circular dichroism, and fluorescence spectra of the AhpF flavin. The N-terminal portion of AhpF can thus be thought of as an appended "redox module" containing an "auxiliary" redox-active disulfide and imparting the ability to this protein to interact with and transfer electrons to AhpC. Interestingly, TrR itself cannot reduce AhpC unless an additional small redox protein, thioredoxin (Tr), is added, and, even then, the electron transfer is relatively inefficient (*6*).

Apart from the additional redox center and 200 extra amino acids at the N-terminus, many spectral and functional features of AhpF are quite similar to those of bacterial TrR. During anaerobic reductive titrations of both proteins, both flavin and disulfide centers are progressively reduced due to similar redox potentials; in wild-type AhpF, both disulfide centers are nonetheless reduced preferentially to the flavin, a feature which is maintained in the $Ser_{129}Ser_{132}$ mutant but not the $Ser_{345}Ser_{348}$ mutant. Reduction of the flavin during equilibrium titrations of AhpF leads first to the formation of large amounts of the one-electron-reduced blue, neutral semiquinone form (over 90%), although, unlike TrR, formation of this species during pyridine nucleotide titrations is not light- and/or EDTA-dependent. Full reduction of the flavin of AhpF is accomplished on further addition of dithionite, but not NADH, even when added in great excess. As was true for bacterial TrR, no thiolate → FAD charge-transfer band around 530 nm was observed during reductive titrations of wild-type AhpF. In contrast, an absorbance band attributable to this species was observed in the $Ser_{135}Cys_{138}$ mutant of TrR, particularly at high pH and in the presence of ammonium chloride (*20*), but no such species was detected in the comparable $Ser_{345}Cys_{348}$ mutant of AhpF under similar conditions. We hypothesize that in AhpF the thiolate form of Cys_{348}, which is presumably present to at least some degree at high pH, is not in a proper orientation with respect to the FAD for charge-transfer interaction. It is likely, however, that this residue, like Cys_{138} of TrR, lies somewhat closer to the C4a position of the flavin than does Cys_{345} (*13*); the corresponding mutants of AhpF and TrR, $Cys_{345}Ser_{348}$ and $Cys_{135}Ser_{138}$, respectively, both exhibit quenching of the FAD fluorescence (*22*). This fluorescence quenching has been attributed in TrR to an effect of the hydroxyl group of Ser_{138} although, in the case of AhpF, if Ser_{348} were responsible for the quenching of the flavin fluorescence, the enhanced fluorescence of the flavin in the $Ser_{345}Ser_{348}$ mutant might not be expected. It is possible that flavin fluorescence is also a function of the conformational state of the enzyme, a situation which would make the interpretation of these fluorescence data considerably more complex.

In considering the role that the N-terminal disulfide center of AhpF plays in catalysis of AhpC reduction, we favor active participation of this redox center in electron transfer between the dithiol form of $Cys_{345}Cys_{348}$ and AhpC, although this need not necessarily be the case. One other member of the larger family of pyridine nucleotide:disulfide oxidoreductases, mercuric reductase, also possesses an auxiliary redox-active disulfide which is at the C-terminus between adjacent residues, Cys_{558} and Cys_{559} (*23*). In this case, reduction of the auxiliary disulfide by the disulfide center proximal to the flavin, $Cys_{135}Cys_{140}$, produces an "activated" enzyme which is capable of binding Hg(II) and catalyzing reduction of the ion to produce Hg^0 without inactivation of the enzyme. This auxiliary dithiol probably persists through the catalytic cycle and is not subject to redox cycling during catalysis. While this latter characteristic could be true of $Cys_{129}Cys_{132}$ in AhpF, as well, there is no clear specialized role for this center as there is for $Cys_{558}Cys_{559}$ in mercuric reductase in assisting in binding and possibly modulating the redox potential of the mercuric ion to favor reduction by the enzyme (*23*). Any activation effect of reduction of the $Cys_{129}Cys_{132}$ center of AhpF would presumably be in optimizing the binding of AhpC, and it seems likely that the electron transfer would also directly involve this redox center rather than only being influenced by conformational changes promoted by the dithiol redox state of $Cys_{129}Cys_{132}$.

A surprising result from these studies has been that the N-terminal redox center can be reduced by the flavin even in the absence of $Cys_{345}Cys_{348}$. This result is quite distinct from those obtained with similar mutants of the homologous protein from *A. xylanus* and with comparable mutants of the more distantly related mercuric reductase enzyme. When Cys_{337} and/or Cys_{340} of the *A. xylanus* protein were mutated, no reduction of the N-terminal disulfide center was observed (*21*). Similarly, the $Ser_{135}Ser_{140}$ mutant of mercuric reductase was unable to reduce the auxiliary disulfide center, $Cys_{558}Cys_{559}$ (*23*). While it is clear that direct reduction of the N-terminal center of AhpF by flavin can occur, it is also notable that this electron transfer is relatively slow, particularly during dithionite titrations, strongly indicating that the normal pathway of electron flow has been disrupted. This result does not, therefore, conflict with the idea that electron transfer during catalysis occurs first from flavin to $Cys_{345}Cys_{348}$, and then from that nascent dithiol to $Cys_{129}Cys_{132}$. Our data also provide additional evidence that the N-terminal disulfide center is able to communicate with flavin at the active site. This evidence derives from the fact that, in the absence of $Cys_{129}Cys_{132}$, charge-transfer interaction between $FADH_2$ and NAD^+ is dramatically decreased, while the absence of $Cys_{345}Cys_{348}$ has no such effect. This result demonstrates that, at the very least, the dithiol form of $Cys_{129}Cys_{132}$ affects the conformation of the flavoprotein, either bringing the nicotinamide ring of NAD^+ into close proximity with the isoalloxazine ring or promoting binding of NAD^+. Indeed,

interaction of $Cys_{129}Cys_{132}$ with flavin and/or $Cys_{345}Cys_{348}$ in dimeric AhpF may result from intersubunit or even interprotein interactions. In mercuric reductase, biochemical as well as structural data from the dimeric protein indicate that the auxiliary disulfide from the C-terminus of one subunit is part of the active site comprised of FAD and $Cys_{135}Cys_{140}$ from the other subunit (*23*, *24*). We are currently pursuing investigations to detect such intersubunit electron transfer in AhpF.

The major disruption of peroxidase activity in the presence of AhpC that occurs on mutation of either disulfide center of AhpF is also substantially mimicked by the effect of these mutations on the catalytic reduction of the chromogenic aryl disulfide DTNB. These results suggest that DTNB as a substrate acts much like AhpC in the mechanism by which it obtains its electrons. Consistent with these results is the demonstration by Schultz et al. (*25*) that the considerable activity of wild-type mercuric reductase with DTNB (440 min^{-1} $dimer^{-1}$) was decreased to 3% in the $Cys_{135}Ser_{140}$ mutant and undetectable in the $Ser_{135}Cys_{140}$ mutant. All three mutants of the *A. xylanus* protein exhibited less than 3% activity with DTNB (*21*). As has been suggested for the *A. xylanus* flavoprotein, the bound flavin of AhpF may be directly responsible for the low level of DTNB reductase activity catalyzed by cysteine mutants of this protein.

Effects of all mutations of AhpF studied herein were quite modest with respect to transhydrogenase and oxidase activities. Our interpretation of these results, supported by earlier work with thiol modifying agents (*4*), is that these two activities are directly mediated by the bound flavin and do not require the presence of any of the catalytic cysteine residues. Relatively subtle effects which cause the variation in these activities to give values from 80 to 230% compared to those of wild-type AhpF are likely caused by changes in the electronic environment of the flavin and/or altered conformational preferences for the different mutants. Indeed, the effect of mutations on the flavin fluorescence is loosely correlated with transhydrogenase activity, with the most fluorescent mutant showing the greatest activity ($Ser_{345}Ser_{348}$) and the least fluorescent mutant exhibiting the lowest transhydrogenase activity ($Cys_{345}Ser_{348}$). In mercuric reductase, the transhydrogenase activities of the two mutants studied, $Cys_{135}Ser_{140}$ and $Ser_{135}Cys_{140}$, were 122 and 43% of wild-type, respectively (*25*). The oxidase activities of these mutants were 425 and 10% of wild-type, respectively, although both transhydrogenase and oxidase activities were substantially lower for mercuric reductase than they are for AhpF (approximately 2 and 1%, respectively, under somewhat different reaction conditions). Oxidase activities for Cys_{337} and/or Cys_{340} mutants of the *A. xylanus* flavoprotein were measured by extrapolation over a range of oxygen and NADH concentrations and found to give essentially identical K_m values for both substrates and a lowered V_{max} value (to about 61%) for only the $Cys_{337}Ser_{340}$ mutant (*21*).

In summary, our data clearly demonstrate the requirement for both redox-active disulfide centers of AhpF in catalysis of AhpC and DTNB reduction, but not in transhydrogenase or oxidase activities. In the absence of one redox-active disulfide center, the other center of AhpF is still capable of being reduced by the bound flavin, although in the $Ser_{345}Ser_{348}$ mutant this process is substantially slower than in either wild-type or $Ser_{129}Ser_{132}$ proteins. The $Cys_{345}Cys_{348}$ center is in close proximity to the flavin of AhpF, similar to the corresponding $Cys_{135}Cys_{138}$ center of TrR, and may transfer electrons to the $Cys_{129}Cys_{132}$ center during electron flow to AhpC and hydroperoxide substrates, although we have yet to clearly establish the active participation of the N-terminal redox center in the catalytic cycle.

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