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MINIREVIEW

AhpF and other NADH:peroxiredoxin oxidoreductases, homologues of low M_r thioredoxin reductase

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A group of bacterial flavoproteins related to thioredoxin reductase contain an additional ≈200-amino-acid domain including a redox-active disulfide center at their N-termini. These flavoproteins, designated NADH:peroxiredoxin oxidoreductases, catalyze the pyridine-nucleotide-dependent reduction of cysteine-based peroxidases (e.g. *Salmonella typhimurium* AhpC, a member of the peroxiredoxin family) which in turn reduce H_2O_2 or organic hydroperoxides. These enzymes catalyze rapid electron transfer ($k_{cat} > 165\ s^{-1}$) through one tightly bound FAD and two redox-active disulfide centers, with the N-terminal-most disulfide center acting as a redox mediator between the thioredoxin-reductase-like part of these proteins and the peroxiredoxin substrates. A chimeric protein with the first 207 amino acids of *S. typhimurium* AhpF attached to the N-terminus of *Escherichia coli* thioredoxin reductase exhibits very high NADPH:peroxiredoxin oxidoreductase and thioredoxin reductase activities. Catalytic turnover by NADH:peroxiredoxin oxidoreductases may involve major domain rotations, analogous to those proposed for bacterial thioredoxin reductase, and cycling of these enzymes between two electron-reduced (EH_2) and four electron-reduced (EH_4) redox states.

Keywords: flavoproteins; peroxiredoxin; oxidoreductases; redox-active disulfide centers; electron transfer proteins; redox mediators; NADH oxidases; AhpF; AhpC; thioredoxin reductase.

RELATIONSHIP BETWEEN BACTERIAL THIOREDOXIN REDUCTASE AND ALKYL HYDROPEROXIDE REDUCTASE F52A PROTEIN (AHPF): STRUCTURES AND ACTIVITIES OF THE PEROXIREDOXIN REDUCTASE FAMILY

A group of bacterial flavoproteins, of which AhpF from *Salmonella typhimurium* was the first-known member [1,2], have evolved from thioredoxin reductase or a common ancestor to specifically reactivate cysteine-based peroxidases such as *S. typhimurium* AhpC through reduction of their active site intersubunit disulfide bonds [3–5]. AhpC homologues, which are ubiquitous, have collectively been designated the peroxiredoxin family [3,6]. AhpF family members are therefore appropriately grouped according to their common activity as NADH:peroxiredoxin oxidoreductases (EC 1.6.4.-) or peroxiredoxin reductases (PrxRs) for short.

PrxRs are related to full-length bacterial thioredoxin reductase throughout about 60% of their sequence at the C-terminal end (≈ 36–40% identity; Fig. 1A). Absolutely conserved between PrxRs and *Escherichia coli* thioredoxin reductase are the redox-active half-cystine residues, within a CXXC motif, located 14 amino acids upstream of the beginning of the pyridine nucleotide binding motif (GXGXXG/A). Appended to the thioredoxin reductase-like region of PrxRs is an N-terminal domain of about 200 amino acids containing an additional redox-active CXXC disulfide center. High resolution structural information about each of these regions in PrxRs is likely to soon be available [7,8] (Z. A. Wood and P. A. Karplus, unpublished results). In the meantime, folding predictions and modeling efforts have fully supported the thioredoxin reductase-like fold of the C-terminal portion of PrxRs [9,10]; a molecular replacement model of the C-terminal portion of *E. coli* AhpF has successfully led to the solution of the structure of a C-terminal fragment of this protein from X-ray crystallographic data as presented in a preliminary report [8]. The N-terminus of PrxRs has only recently been recognized, using the FFAS fold prediction method [11], to contain a tandem repeat of two thioredoxin-like folds of which only the second retains its redox-active disulfide center [10]. Preliminary crystallographic analysis supports this prediction (Z. A. Wood and P. A. Karplus, unpublished results).

All PrxR family members identified to date have been found in eubacteria (Table 1). A number of PrxRs and/or their peroxiredoxin substrates have been implicated in the protection of the bacteria against oxidative stress [12–20], osmotic stress [21] or organic solvents [22,23]. Most of those which were initially identified through functional analyses were originally noted for their NADH oxidase [24–28] or NADH dehydrogenase [29,30] activities and only subsequently recognized for their alkyl hydroperoxide reductase activity in the presence of AhpC homologues (Table 1) [31–35]. The NADH oxidase activity, which is higher in some PrxRs from Gram-positive organisms,

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Abbreviations: PrxR, NADH:peroxiredoxin oxidoreductases; Prx, peroxiredoxin; FFAS, fold and function assignment system; DTNB, 5,5′-dithiobis(2-nitrobenzoate); TNB, 2-nitro-5-thiobenzoate.

Note: a web page is available at http://www.wfubmc.edu/biochem/faculty/Poole/poole.html

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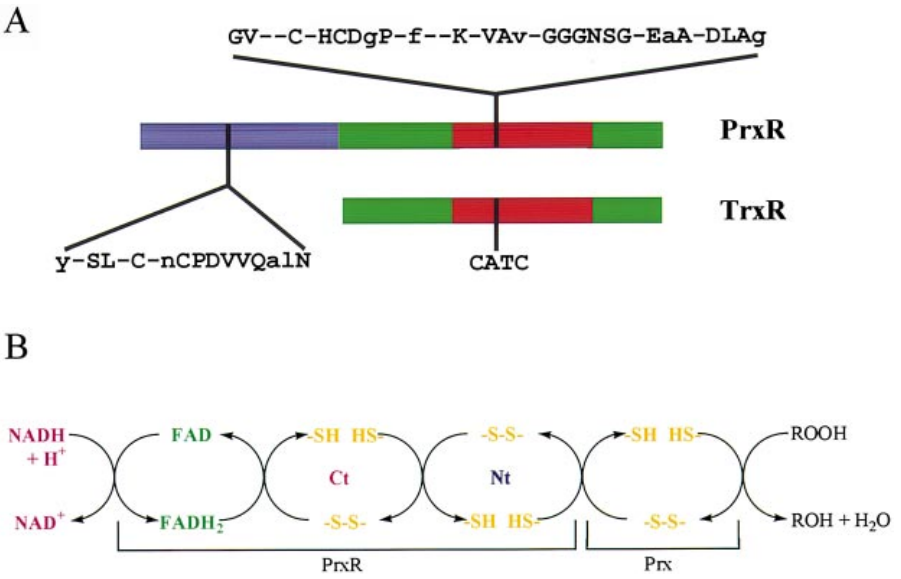

Fig. 1. Redox centers of peroxiredoxin reductases (PrxRs): primary structure compared with *E. coli* thioredoxin reductase (TrxR) and sequence of electron transfers. Shown in green in (A) are the noncontiguous portions of the flavin-binding domains. The pyridine nucleotide binding domains are shown in red, and the N-terminal domain of PrxRs such as that of *S. typhimurium* AhpF is shown in blue. Consensus sequences surrounding the redox-active half-cystine residues of PrxRs are shown, with upper case letters indicating sites of absolute conservation and lower case letters showing positions of identity in 16 of the 17 homologues identified to date. Full sequence alignments of 12 of the homologues have been presented elsewhere [35]. During the catalytic cycle of alkyl hydroperoxide reductases (B), two electrons from NADH [accompanied by two H^+ are transferred among three redox centers in PrxR proteins through the bound flavin, the C-terminal disulfide center (Ct), and the N-terminal disulfide center (Nt)], to the redox-active intersubunit disulfide bond of peroxiredoxins (Prx), then to the hydroperoxide substrate.

is significantly enhanced by the addition of free FAD; this effect results from the nonenzymatic autoxidation of free reduced FAD generated through the dehydrogenase activity of PrxRs [25,27,33,35]. While the turnover values for these enzymes with oxygen (in air-saturated buffers at 25 °C) and excess NADH of about 1–15 s^{-1} are relatively high for flavoprotein dehydrogenases, turnover values under similar conditions with saturating peroxiredoxins as electron acceptors

Table 1. NADH:peroxiredoxin oxidoreductases. Organisms with NADH:peroxiredoxin oxidoreductase-encoding structural genes were identified through BLAST searches [58] of GenBank and other databases containing genomic information for a large number of organisms. Abbreviations for activities indicate that peroxidase in the presence of a peroxiredoxin substrate (P), DTNB reductase (DR), NADH oxidase (O) and/or dehydrogenase activity with 2,6-dichloroindophenol (DH) was reported. Sequences from the various organisms can be downloaded from GenBank using the accession number or from Washington University Genome Sequencing Center (http://genome.wustl.edu/gsc/), The Institute for Genomic Research (TIGR, http://www.tigr.org/tdb/), University of Washington Genome Center (http://www.genome.washington.edu/UWGC/), or University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu/), as indicated.

Organism	Accession number	Activity demonstrated	References
Proteobacteria			
Salmonella typhimurium	P19480	P, DR, O, DH	[1,2,4,12,35]
Escherichia coli	P35340	P, O, DH	[1,12,22,59]
Klebsiella pneumoniae	Contig 1065		Washington University
Shewanella putrefaciens	Contig 5551		TIGR
Xanthomonas campestris pv. phaseoli	O06465		[17,18]
Pseudomonas putida	O82864		[23] (F. Fukumori and K. Horikoshi, unpublished results)
Pseudomonas aeruginosa	RPA00477		University of Washington
Firmicutes			
Streptococcus mutans	AB010712.1	P, DR, O, DH	[27,28,35]
Streptococcus pyogenes	Contig 1		University of Oklahoma
Amphibacillus xylanus	AB018435.1	P, DR, O, DH	[25,26,31]
Bacillus alcalophilus	P26829	P, O, DH	[29,30,34]
Bacillus subtilis	P42974		[15,16]
Bacillus anthracis	Contig 601		TIGR
Staphylococcus aureus	O05204		[21]
Thermus/Deinococcus group			
Thermus aquaticus	AF276071	P, DR, O, DH	[33,38,48]
CFB group			
Bacteroides fragilis	AAD52148		[20]
Porphyromonas gingivalis	RPG01526		TIGR

are much greater (165–250 s^{-1}) [31,34,35]. Both activities may be of relevance under physiological conditions within aerobically growing bacteria.

Like thioredoxin reductase, PrxRs catalyze the reduction of a redox-active disulfide bond in a substrate protein through electron transfers among pyridine nucleotide, FAD, and redox-active disulfide centers (Fig. 1B). The disulfide-reducing activity of PrxRs can be mimicked using a small disulfide substrate, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), which generates a chromophoric product (Table 1). DTNB is not directly reduced by low M_r thioredoxin reductase, but instead requires the presence of thioredoxin as a redox mediator [36]. Rapid reduction of both peroxiredoxins and DTNB requires both the C-terminal and N-terminal redox-active disulfide centers to be intact as demonstrated for PrxRs from *S. typhimurium* and *Amphibacillus xylanus* [10,31,37]. Oxidase activity, on the other hand, is not significantly affected by mutagenesis of any of the four catalytic cysteine residues. Transhydrogenase activity, which is also quite high in PrxRs, is similarly unaffected by cysteine mutagenesis. The N-terminal 'appendage' of PrxRs containing the additional CXXC motif (relative to thioredoxin reductase) was thus implicated as an essential player in the NADH:peroxiredoxin oxidoreductase catalytic cycle through cysteine mutagenesis [31,37] and protein truncation [10].

SPECTROSCOPIC AND KINETIC PROPERTIES OF PEROXIREDOXIN REDUCTASES

That the N-terminal disulfide center of PrxRs is redox-active has been demonstrated for *S. typhimurium*, *A. xylanus*, *Streptococcus mutans* and *Thermus aquaticus* proteins, which require ≈3 eq. dithionite per FAD for full reduction (Fig. 2A) [5,26,35,38]. Removal of the N-terminal domain through truncation or substitution of the N-terminal cysteine residues by serine residues in *S. typhimurium* AhpF yielded proteins with similar spectroscopic properties above 300 nm but requiring only ≈2 eq. dithionite per FAD for full reduction [10,37]. During reductive titrations with dithionite and NADH, PrxRs exhibit a prominent absorbance band centered around 580 nm, attributed to the stabilization of the blue, neutral flavin semiquinone (Fig. 2A) [5,26,33,35,38]. This species is unlikely to be of catalytic significance due to its relatively slow formation. Neutral semiquinone formation is also a characteristic of *E. coli* thioredoxin reductase [39]. The stabilization of the protonated form of the semiquinone is diagnostic for flavin dehydrogenases, whereas flavin-containing oxidases and oxygenases typically stabilize the unprotonated red anionic form of flavin semiquinone in cases where this species is observed [40,41].

Reductive titrations of PrxRs with NADH also require > 2.5 eq. reductant per FAD; in this case, however, reduction of the flavin does not go to completion, demonstrating that NADH is not an effective reductant of FADH· [5,26,33,35]. The most striking difference between NADH and dithionite titrations of PrxRs is the development of a very long wavelength absorbance band (extending beyond 900 nm) in the presence of excess pyridine nucleotide (Fig. 2B). This absorbance band is the result of a charge transfer interaction between reduced flavin and NAD^+ [42] and is particularly prominent in NADH titrations of PrxRs from Gram-positive organisms [5,26,33,35]. A similar absorbance band is observed during enzyme-monitored turnover studies of thioredoxin reductase with NADPH and thioredoxin [43], and of *S. typhimurium* AhpF

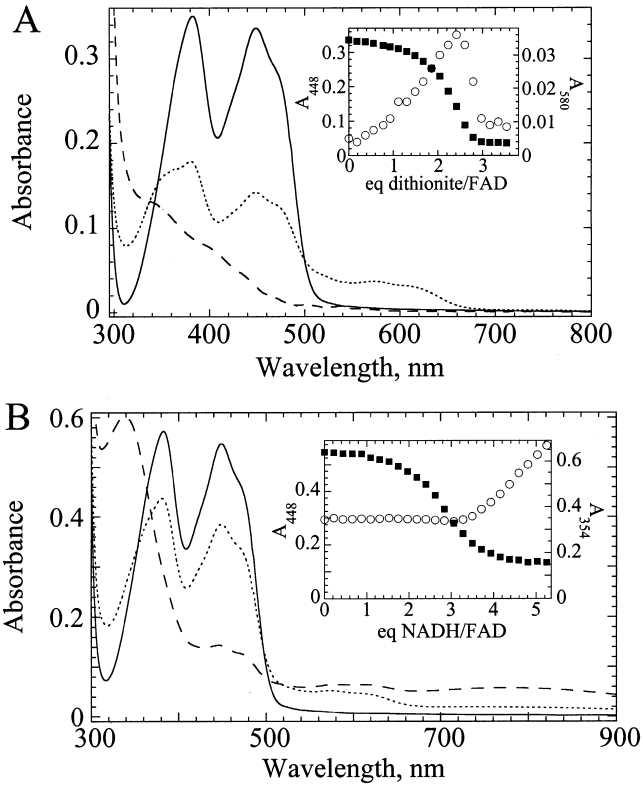

Fig. 2. Anaerobic dithionite (A) and NADH (B) titrations of Nox-1, the PrxR from *Streptococcus mutans*. Spectra shown in (A) were obtained after the addition of 0 (solid line), 2.42 (dotted line) and 3.0 (dashed line) dithionite/FAD. The inset shows absorbance changes at 448 nm (closed squares) and 580 nm (open circles) during the course of the titration. Spectra shown in (B) were obtained after the addition of 0 (solid line), 2.84 (dotted line) and 4.81 (dashed line) eq. NADH per FAD. The inset shows absorbance changes at 448 nm (closed squares) and 354 nm (open circles) during the course of the titration. Details of these experiments are described elsewhere [35].

with NADH and AhpC (L. B. Poole and M. Li Calzi, unpublished results); redox species with such a reduced flavin → oxidized pyridine nucleotide interaction may therefore be of catalytic significance for this superfamily of flavoprotein reductases.

Steady state assays of PrxRs in the presence of NADH, AhpC and hydrogen peroxide or cumene hydroperoxide have indicated turnover values for these proteins of > 165 s^{-1} (except for *T. acquaticus* PrxR, which exhibits considerably lower activity with its corresponding Prx [38]). These high values are remarkable given the multiple redox centers in these proteins which must undergo electron transfer. By comparison, *E. coli* thioredoxin reductase under similar conditions exhibits a k_{cat} of about 38 s^{-1} [43]. PrxRs have K_m values for NADH that are too low to be readily determined by the standard methods [31,35]. In the presence of excess NADH and peroxide, K_m values for AhpC are around 15 μM [34,35,44]. Such a K_m value is consistent with the fact that peroxiredoxins such as AhpC have been characterized as abundant proteins in a number of organisms [3]. Interestingly, addition of ammonium sulfate to assay buffers increases the rate of peroxidase, DTNB reductase and transhydrogenase activities under standard assay conditions [4,31,35,38]. Ammonium sulfate has been shown to decrease the K_m values for NADH (using DTNB reductase activities) [4] and for AhpC (using peroxidase assays) [31]. The increased

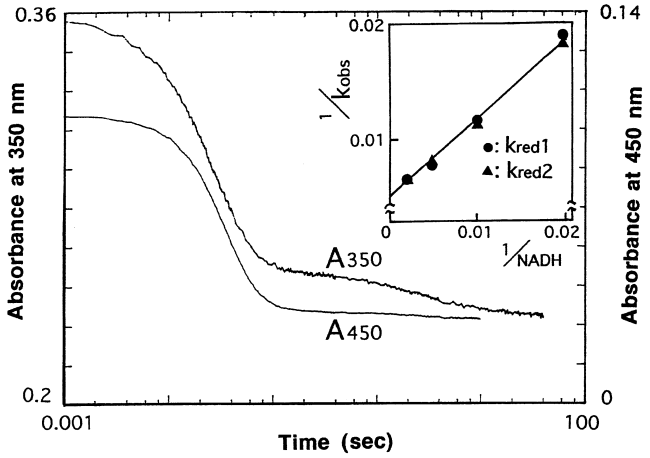

Fig. 3. Time course for the reduction of the PrxR from *Amphibacillus xylanus* by NADH. The protein was mixed anaerobically with a 6.7-fold excess of NADH at 25 °C. Absorbance changes were monitored at 350 and 450 nm. The inset shows a double reciprocal plot of the two fast k_{obs} values (s) as a function of NADH concentration (μM^{-1}) at 350 or 450 nm. Reprinted with permission from the *Journal of Biological Chemistry* [45].

ionic strength of ammonium sulfate-containing buffers is responsible for this effect and may promote the protein–protein interaction required for turnover. Although several of the Gram-positive PrxRs exhibit absolute specificity for NADH [25,27,33,35], AhpF from *S. typhimurium* can also use NADPH as its reducing substrate, albeit with a much higher K_m (as determined by DTNB reductase assays) [4].

Rapid reaction kinetic studies have been reported for the PrxR from *A. xylanus* and are summarized in Fig. 3 [45]. During reduction of the enzyme by excess NADH, three phases of flavin reduction were observed by stopped-flow spectroscopy. The first two phases, each showing NADH dependence and limiting rates of about 200 s^{-1}, corresponded to the oxidation of about 1 eq. NADH per FAD during each phase. Essentially no change in flavin absorbance was observed during the first phase, indicating that the electrons were quickly transferred to one (or both) of the disulfide redox centers during this phase. Major loss of flavin absorbance occurred during the second phase of reduction. Only small, NADH-independent spectral changes and the oxidation of about 0.6 eq. NADH per FAD occurred during the third phase which, at 0.3 s^{-1}, was not fast enough to be of catalytic importance (k_{cat} under these conditions and in the presence of saturating *S. typhimurium* AhpC was about 180 s^{-1}). These results indicated rapid reduction of the active site disulfide(s) by reduced flavin, limited by the rate of transfer of electrons to flavin from NADH.

In the absence of the C-terminal active site cysteine residues (in the C337S/C340S mutant of the *A. xylanus* PrxR), only a single NADH-dependent rate was observed, at 285 s^{-1}, and this phase accounted for oxidation of most of a single equivlent of NADH; two other minor phases of flavin reduction and NADH oxidation in this experiment proceeded at rates of 8 s^{-1} or less. In this work [45] and previous reductive titrations with the C337S/C340S mutant [46], the C-terminal disulfide center was shown to be required for reduction of the N-terminal redox center. Curiously, the N-terminal disulfide center could still be reduced, albeit relatively slowly, in reductive titrations of the corresponding mutant of *S. typhimurium* AhpF (C345S/C348S) [37].

Preliminary stopped-flow studies of the *S. typhimurium* enzyme have indicated an extremely rapid rate of partial flavin reduction (> 1000 s^{-1}) and the oxidation of more than 1 eq. NADH per FAD in the dead time of the instrument (≈ 1.5 ms), even at 5 °C (L. B. Poole and M. Li Calzi, unpublished results). Although the PrxRs from *A. xylanus* and *S. typhimurium* have rather different properties at the level of their rapid reaction kinetics, both are likely to undergo the same pathway of electron transfer, i.e. from NADH to the flavin, from the reduced flavin to the C-terminal (proximal) disulfide center, and from this center to the N-terminal (distal) redox-active disulfide. While the slow third phase of flavin reduction and NADH oxidation in the PrxR of *A. xylanus* (attributed to the generation of six electron-reduced enzyme limited by the rate of electron transfer between the C-terminal and N-terminal redox centers) was too slow for catalysis, this result does not necessarily preclude the direct participation of the N-terminal redox center in catalysis; in the presence of substrate (AhpC) this rate could be increased, and/or the fully reduced enzyme need not be a catalytic species.

ROLE OF THE N-TERMINAL DOMAIN IN AHPC REDUCTION AND COMPLEX FORMATION: GENERATION OF A CHIMERIC PROTEIN WITH BACTERIAL THIOREDOXIN REDUCTASE

Whether the N-terminal domain of PrxRs plays a direct, intermediary role in transferring electrons from the C-terminal redox centers of these proteins to their AhpC substrates has been addressed in a variety of ways with the *S. typhimurium* AhpF protein. As mentioned above, all four cysteine residues and an intact N-terminal domain are required for NADH:peroxiredoxin oxidoreductase activity in the *S. typhimurium* and *A. xylanus* PrxR proteins [10,31,37,47]. That the N-terminal domain is the direct electron donor to AhpC was recently demonstrated by stopped-flow kinetic studies using separately expressed fragments of *S. typhimurium* AhpF [10]. In these studies, the thioredoxin reductase-like C-terminal fragment beginning at residue 208 of AhpF, designated F[208–521], was independently expressed and purified, as was the N-terminal domain up to residue 202 (designated F[1–202]). These two fragments possessed many of the structural and redox properties of their parent protein, but interacted relatively slowly for electron transfer, with a second order rate constant of about 4×10^4 $M^{-1} \cdot s^{-1}$ at 25 °C. Peroxidase activity was therefore not restored to any significant level on mixing of the two fragments in the presence of AhpC. To directly assess the ability of each fragment to reduce AhpC, a new substrate was designed that contained a fluorescein derivative linked through a disulfide bond to Cys165 of the C46S mutant of *S. typhimurium* AhpC (Fig. 4). Intact AhpF reduced this disulfide bond in the presence of excess NADH at rates similar to catalysis with wild-type AhpC. Under identical conditions, F[208–521] exhibited < 0.1% of the rate of fluorophore release from this engineered substrate compared with intact AhpF. Reduced F[1–202], on the other hand, reduced the disulfide bond of this engineered substrate with a bimolecular rate constant very similar to the catalytic efficiency observed during turnover of this substrate with intact AhpF [10]. Therefore, only the N-terminal redox center can rapidly reduce AhpC, leading to the conclusion that this center directly mediates electron transfer from the C-terminal part of AhpF to AhpC during catalysis.

During electron transfer between PrxRs and peroxiredoxins, the two proteins must come together and be transiently linked via a covalent bond for dithiol/disulfide interchange to occur

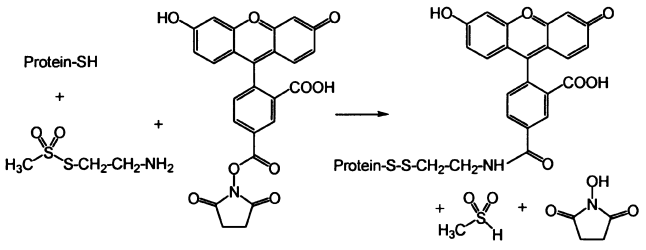

Fig. 4. AhpC substrate analogue with fluorescein attached via a disulfide bond. Succidimidyl ester-linked fluorescein was reacted with aminoethyl methane thiosulfonate, then with the free thiol of the C46S mutant of AhpC. The resulting disulfide-linked fluorophore exhibited extensive quenching of the fluorescence when covalently bound to the protein. Release of the fluorophore by reduction with reduced AhpF or F[1–202] resulted in a large increase in fluorescence [10].

between the two, analogous to the direct interaction between thioredoxin reductase and thioredoxin proteins. Although there are numerous citations concerning PrxRs which erroneously designate these proteins as 'subunits' of an alkyl hydroperoxide reductase holoenzyme, in fact no evidence for complex formation between wild-type PrxR and peroxiredoxin proteins has been obtained (except in the case of the *T. aquaticus* proteins [48]). AhpF of *S. typhimurium* is dimeric, as shown by analytical ultracentrifugation [10], and AhpC proteins from *A. xylanus* and *S. typhimurium* are decamers [49] (Z. A. Wood, P. A. Karplus, R. R. Hantgan and L. B. Poole, unpublished results). In all cases (with the exception of the native *T. aquaticus* proteins), these proteins have been readily purified as separate proteins from bacterial cytosols.

In order to mimic and stabilize the transiently linked catalytic complex between AhpF and AhpC of *S. typhimurium*, single cysteine mutants of each protein were combined following oxidation of one of the two proteins by DTNB [47] as was previously performed with thioredoxin reductase and thioredoxin from *E. coli* [50,51]. Only one of the four single cysteine mutants of AhpF, C132S, reacted with oxidized AhpC mutants (primarily with C46S) to form a higher molecular mass complex as assessed by SDS/PAGE. This interaction, as analyzed by stopped-flow spectroscopy, occurs with a second order rate constant quite similar to the catalytic efficiency of the wild-type proteins as assessed by steady state assays. As with covalently linked thioredoxin reductase–thioredoxin complexes, reduced pyridine nucleotide cannot reduce the interprotein disulfide bond, suggesting that the linkage of a large protein to the N-terminus prevents this part of AhpF from moving into close enough proximity of the C-terminal redox center(s) for electron transfer to occur. These results suggest that it is the thiol(ate) of Cys129 in the N-terminal domain of *S. typhimurium* AhpF that acts as the nucleophile in the attack of the active site disulfide bond in AhpC during electron transfer between the two proteins. Small amounts of a similar high-molecular mass complex formed between the C131S mutant of the PrxR from *A. xylanus* and the corresponding wild-type AhpC were also detected by Western blotting of bacterial extracts from this organism [52].

Because of the homology of the C-terminal part of PrxRs with bacterial thioredoxin reductases and the demonstrated ability of *E. coli* thioredoxin reductase to reduce F[1–202] derived from the *S. typhimurium* protein [10], a chimeric protein between *E. coli* thioredoxin reductase and residues 1–207 of *S. typhimurium* AhpF was constructed to assess the ability of this protein to replace AhpF in catalysis of AhpC-mediated peroxide reduction. Indeed, the AhpF-derived N-terminal redox center was reduced by the thioredoxin reductase part of the chimeric molecule during anaerobic dithionite or NADPH titrations, and the chimeric protein exhibited a level of catalytic efficiency in peroxidase assays with NADPH and AhpC nearly identical to that of AhpF itself [53]. Surprisingly, this chimeric protein also retained its thioredoxin reductase activity indicating that the attachment of the N-terminal domain from AhpF to thioredoxin reductase did not block the active site of thioredoxin reductase. The ability of the N-terminus from AhpF to impart AhpC-reducing activity to thioredoxin reductase in this chimera is in complete agreement with the direct role this N-terminal domain plays in mediating electron transfer between redox centers in AhpF and AhpC as elucidated by the experiments summarized above.

HYPOTHESIZED CONFORMATIONAL CHANGES, INTERSUBUNIT VERSUS INTRASUBUNIT ELECTRON TRANSFERS, AND REDOX STATES INVOLVED IN CATALYSIS BY PEROXIREDOXIN REDUCTASES

As in bacterial thioredoxin reductase, the C-terminal redox-active disulfide center of PrxRs resides in the pyridine nucleotide binding domain and must interact with flavin for electron transfer on the same (*re*) face of the isoalloxazine ring as does the pyridine nucleotide [54]. The relative positions of these redox centers as determined by X-ray crystallographic studies of thioredoxin reductase from *E. coli*, with the redox-active disulfide center proximal to the flavin and the nicotinamide ring ≈ 17 Å away, has led to the proposal that an alternate conformation of the enzyme, with the nicotinamide ring stacked over the isoalloxazine ring and the dithiol/disulfide center rotated to a more exposed position, is also involved in catalysis [54,55]. In PrxR proteins, the alternating interactions of redox centers should be even more complex given the additional domain and redox center that participate in catalysis. It is nonetheless possible to envision all electron transfers required for catalysis as occurring during cycling of these enzymes between two conformational states. In one state, analogous to that observed in crystals of thioredoxin reductase, the C-terminal disulfide center would be in a position to receive electrons from reduced flavin, and the redox center in the N-terminus, presumably in its reduced form, would be rotated away from this part of the molecule and into an accessible position for electron transfer to AhpC (Fig. 5). In the other state, corresponding to the hypothesized conformation of thioredoxin reductase, the nicotinamide and isoalloxazine rings would be close to one another to permit hydride transfer between the reduced pyridine nucleotide and the flavin, and the C-terminal and N-terminal dithiol/disulfide centers would be close to one another to permit electron transfer from the C-terminal dithiol to the N-terminal disulfide. Maximum efficiency of a catalytic cycle would be achieved if only two conformational changes were required per catalytic cycle as described in more detail below.

As with low M_r thioredoxin reductase, each molecule of AhpF from *S. typhimurium* and *E. coli* is composed of two subunits in an antiparallel orientation (Z. A. Wood and P. A. Karplus, unpublished results) [8]. The N-terminal domain is attached via a flexible linker (there is a proteolytically sensitive site between residues 202 and 203 [5]) to the end of the flavin binding domain that is farthest from the pyridine nucleotide binding domain. This suggests that, depending on the length and flexibility of the linker, the redox center in the N-terminus

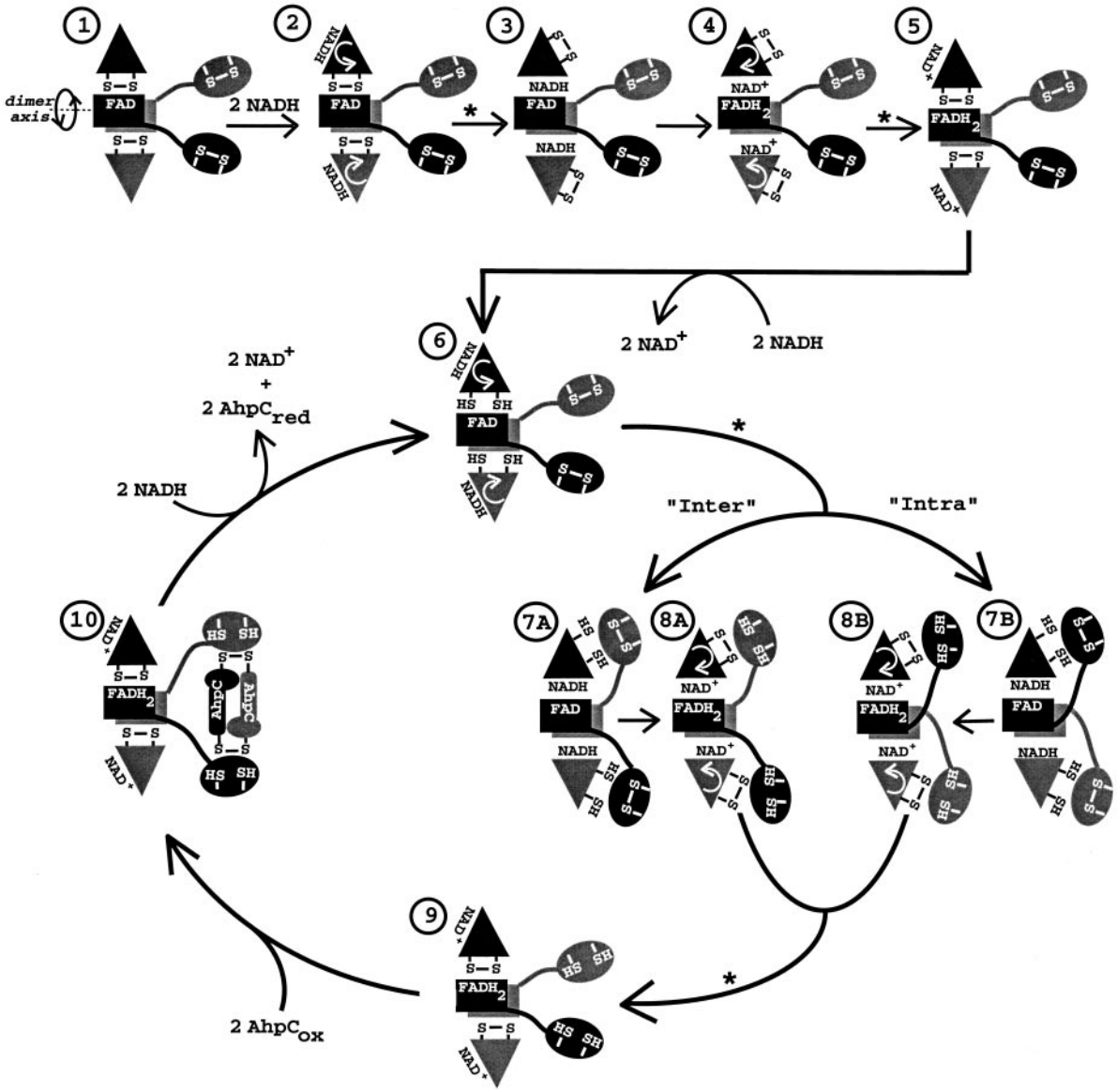

Fig. 5. Hypothesized priming and catalytic cycle of NADH:peroxiredoxin oxidoreductases such as *S. typhimurium* AhpF in the presence of excess NADH and oxidized peroxiredoxin (AhpC). AhpF is depicted as a 'head-to-tail' dimer oriented with the twofold axis in the plane of the page and the individual subunits distinguished by color (black vs. gray). The pyridine nucleotide binding domain (triangle) is shown with NADH or NAD^+ bound and an oxidized (SS) or reduced [$(SH)_2$] Cys345–Cys348 redox center. The redox state of the flavin bound to the flavin binding domain (rectangle) is represented by FAD or $FADH_2$. The disulfide center (Cys129–Cys132) in the N-terminal domain of AhpF (oval attached via a flexible linker) is also depicted in oxidized (SS) or reduced [$(SH)_2$] forms. The simplified priming and turnover steps are depicted as synchronous events in each part of the dimer (two turnovers per dimer per cycle) and alternate paths illustrate the possible intersubunit electron transfer (species **7A** → **8A**) or intrasubunit transfer (species **7B** → **8B**). For simplicity, AhpC is depicted as a single dimer reacting with both N-terminal domains of AhpF in a single step: the oxidized form (SS; $AhpC_{OX}$) reacts with species **10** to yield the reduced form ($AhpC_{RED}$). Steps involving conformational changes are indicted by asterisks. Actual proton transfers and ionization states are not intended to be accurate in this scheme.

of AhpF might approach either the C-terminal redox-active disulfide center in the other subunit or the corresponding redox center in its own subunit [10]. Based on structural considerations, intersubunit electron transfer was previously hypothesized for *S. typhimurium* AhpF [10,37]. However, very recent data using heterodimeric constructs of wild-type and mutant AhpF proteins favors intrasubunit electron transfer during catalysis by AhpF (C. M. Reynolds and L. B. Poole, unpublished results).

Taking all these hypotheses into account, a minimal mechanism for turnover of PrxRs, starting with priming of these enzymes by 1 eq. NADH per subunit, has been proposed (Fig. 5) [10]. This proposal includes coordinated rotations by both the pyridine nucleotide and N-terminal domains to alternate between interacting pairs of redox centers, and the cycling of these proteins between their two electron-reduced (EH_2) and four electron-reduced (EH_4) redox forms during rapid turnover [9,10,47]. An alternative hypothesis put forth by the authors of the rapid reaction kinetic studies of *A. xylanus* PrxR proposes the cycling of that enzyme through oxidized (E) and EH_2 states during catalysis [45]. The hypothesis outlined in Fig. 5 involving the generation of EH_2 from E as a priming step, and cycling occurring between EH_2 and EH_4 is proposed as the most efficient manner through which only two

conformational changes could be required to effect catalytic turnover at a high rate. This view of catalysis also portrays turnover by PrxRs as a combination of domain rotation and mediation of electron transfer by an additional redox carrier, each suggested as individual solutions to accomplish turnover for the two very different types of thioredoxin reductases, from bacterial and eukaryotic sources, that have been characterized [54]. It is interesting to note that in AhpC-reducing systems other than the PrxRs of eubacteria, at least two redox mediators (e.g. thioredoxin reductase plus thioredoxin in yeast [56], or trypanothione reductase plus trypanothione plus tryparedoxin in *Crithidia fasciculata* [57]) are required to carry out the analogous role of the individual PrxR proteins. This family of proteins may have evolved, then, as a way to increase specificity and efficiency of AhpC reduction in these organisms.

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REFERENCES

1. Jacobson, F.S., Morgan, R.W., Christman, M.F. & Ames, B.N. (1989) An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. Purification and properties. *J. Biol. Chem.* **264**, 1488–1496.
2. Tartaglia, L.A., Storz, G., Brodsky, M.H., Lai, A. & Ames, B.N. (1990) Alkyl hydroperoxide reductase from *Salmonella typhimurium*. Sequence and homology to thioredoxin reductase and other flavoprotein disulfide oxidoreductases. *J. Biol. Chem.* **265**, 10535–10540.
3. Chae, H.Z., Robison, K., Poole, L.B., Church, G., Storz, G. & Rhee, S.G. (1994) Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proc. Natl Acad. Sci. USA* **91**, 7017–7021.
4. Poole, L.B. & Ellis, H.R. (1996) Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 1. Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. *Biochemistry* **35**, 56–64.
5. Poole, L.B. (1996) Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 2. Cystine disulfides involved in catalysis of peroxide reduction. *Biochemistry* **35**, 65–75.
6. Kang, S.W., Chae, H.Z., Seo, M.S., Kim, K., Baines, I.C. & Rhee, S.G. (1998) Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. *J. Biol. Chem.* **273**, 6297–6302.
7. MacSweeney, A., D'Arcy, A., Higgins, T.M., Mayhew, S.G., Toomey, D. & Walsh, M.A. (1999) Crystallization and preliminary crystallographic analysis of an NADH oxidase that functions in peroxide reduction in *Thermus aquaticus* YT-1. *Acta Crystallogr.* **D55**, 297–298.
8. Bieger, B. & Essen, L.O. (2000) Crystallization and preliminary X-ray analysis of the catalytic core of the alkylhydroperoxide reductase component AhpF from *Escherichia coli*. *Acta Crystallogr.* **D56**, 92–94.
9. Poole, L.B. (1997) The *Salmonella typhimurium* alkyl hydroperoxide reductase enzyme system. In *Flavins and Flavoproteins 1996* (Stevenson, K.J., Massey, V. & Williams, C.H. Jr, eds), pp. 751–760. University of Calgary Press, Calgary, Canada.
10. Poole, L.B., Godzik, A., Nayeem, A. & Schmitt, J.D. (2000) AhpF can be dissected into two functional units; tandem repeats of two thioredoxin-like folds in the N-terminus mediate electron transfer from the thioredoxin reductase-like C-terminus to AhpC. *Biochemistry* **39**, 6602–6615.
11. Rychlewski, L., Jaroszewski, L., Li, W. & Godzik, A. (2000) Comparison of sequence profiles. Strategies for structural predictions using sequence information. *Protein Sci.* **9**, 232–241.
12. Storz, G., Jacobson, F.S., Tartaglia, L.A., Morgan, R.W., Silveira, L.A. & Ames, B.N. (1989) An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: genetic characterization and cloning of. *Ahp. J. Bacteriol.* **171**, 2049–2055.
13. Farr, S.B. & Kogoma, T. (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.* **55**, 561–585.
14. Hartford, O.M. & Dowds, B.C. (1994) Isolation and characterization of a hydrogen peroxide resistant mutant of *Bacillus subtilis*. *Microbiology* **140**, 297–304.
15. Antelmann, H., Engelmann, S., Schmid, R. & Hecker, M. (1996) General and oxidative stress responses in *Bacillus subtilis*: cloning, expression and mutation of the alkyl hydroperoxide reductase operon. *J. Bacteriol.* **178**, 6571–6578.
16. Bsat, N., Chen, L. & Helmann, J.D. (1996) Mutation of the *Bacillus subtilis* alkyl hydroperoxide reductase (*ahpCF*) operon reveals compensatory interactions among hydrogen peroxide stress genes. *J. Bacteriol.* **178**, 6579–6586.
17. Loprasert, S., Atichartpongkun, S., Whangsuk, W. & Mongkolsuk, S. (1997) Isolation and analysis of the *Xanthomonas* alkyl hydroperoxide reductase gene and the peroxide sensor regulator genes *ahpC* and *ahpFoxyRorfX*. *J. Bacteriol.* **179**, 3944–3949.
18. Mongkolsuk, S., Loprasert, S., Whangsuk, W., Fuangthong, M. & Atichartpongkun, S. (1997) Characterization of transcription organization and analysis of unique expression patterns of an alkyl hydroperoxide reductase C gene (*ahpC*) and the peroxide regulator operon *ahpF-oxyR-orfX* from *Xanthomonas campestris* pv. phaseoli. *J. Bacteriol.* **179**, 3950–3955.
19. Higuchi, M., Yamamoto, Y., Poole, L.B., Shimada, M., Sato, Y., Takahashi, N. & Kamio, Y. (1999) Functions for two types of NADH oxidases in energy metabolism and oxidative stress of *Streptococcus mutans*. *J. Bacteriol.* **181**, 5940–5947.
20. Rocha, E.R. & Smith, C.J. (1999) Role of the alkyl hydroperoxide reductase (*ahpCF*) gene in oxidative stress defense of the obligate anaerobe *Bacteroides fragilis*. *J. Bacteriol.* **181**, 5701–5710.
21. Armstrong-Buisseret, L., Cole, M.B. & Stewart, G.S.A.B. (1995) A homologue to the *Escherichia coli* alkyl hydroperoxide reductase AhpC is induced by osmotic upshock in *Staphylococcus aureus*. *Microbiology* **141**, 1655–1661.
22. Ferrante, A.A., Augliera, J., Lewis, K. & Klibanov, A.M. (1995) Cloning of an organic solvent-resistance gene in *Escherichia coli*: the unexpected role of alkylhydroperoxide reductase. *Proc. Natl Acad. Sci. USA* **92**, 7617–7621.
23. Fukumori, F., Hirayama, H., Takami, H., Inoue, A. & Horikoshi, K. (1998) Isolation and transposon mutagenesis of a *Pseudomonas putida* KT2442 toluene-resistant variant: involvement of an efflux system in solvent resistance. *Extremophiles* **2**, 395–400.
24. Cocco, D., Rinaldi, A., Savini, I., Cooper, J.M. & Bannister, J.V. (1988) NADH oxidase from the extreme thermophile *Thermus aquaticus* YT-1. Purification and characterisation. *Eur. J. Biochem.* **174**, 267–271.
25. Niimura, Y., Ohnishi, K., Yarita, Y., Hidaka, M., Masaki, H., Uchimura, T., Suzuki, H., Kozaki, M. & Uozumi, T. (1993) A flavoprotein functional as NADH oxidase from *Amphibacillus xylanus* Ep01: purification and characterization of the enzyme and structural analysis of its gene. *J. Bacteriol.* **175**, 7945–7950.
26. Ohnishi, K., Niimura, Y., Yokoyama, K., Hidaka, M., Masaki, H., Uchimura, T., Suzuki, H., Uozumi, T., Kozaki, M., Komagata, K. & Nishino, T. (1994) Purification and analysis of a flavoprotein functional as NADH oxidase from *Amphibacillus xylanus* overexpressed in *Escherichia coli*. *J. Biol. Chem.* **269**, 31418–31423.
27. Higuchi, M., Shimada, M., Yamamoto, Y., Hayashi, T., Koga, T. & Kamio, Y. (1993) Identification of two distinct NADH oxidases corresponding to H_2O_2-forming oxidase and H_2O-forming oxidase induced in *Streptococcus mutans*. *J. Gen. Microbiol.* **139**, 2343–2351.
28. Higuchi, M., Shimada, M., Matsumoto, J., Yamamoto, Y., Rhaman, A. & Kamio, Y. (1994) Molecular cloning and sequence analysis of the

gene encoding the H_2O_2-forming NADH oxidase from *Streptococcus mutans*. *Biosci. Biotech. Biochem.* **58**, 1603–1607.
29. Xu, X., Kanaya, S., Koyama, N., Sekiguchi, T., Nosoh, Y., Ohashi, S. & Tsuda, K. (1989) Tryptic digestion of NADH dehydrogenase from alkalophilic *Bacillus*. *J. Biochem.* **105**, 626–632.
30. Xu, X., Koyama, N., Cui, M., Yamagishi, A., Nosoh, Y. & Oshima, T. (1991) Nucleotide sequence of the gene encoding NADH dehydrogenase from an alkalophile, *Bacillus* sp. strain YN-1. *J. Biochem.* **109**, 678–683.
31. Niimura, Y., Poole, L.B. & Massey, V. (1995) *Amphibacillus xylanus* NADH oxidase and *Salmonella typhimurium* alkyl hydroperoxide reductase flavoprotein component show extremely high scavenging activity for both alkyl hydroperoxide and hydrogen peroxide in the presence of *S. typhimurium* alkyl hydroperoxide reductase 22-kDa protein component. *J. Biol. Chem.* **269**, 25645–25650.
32. Poole, L.B., Shimada, M. & Higuchi, M. (1997) NADH oxidase-1 and a second component encoded upstream of *nox1* comprise an alkyl hydroperoxide reductase system in *Streptococcus mutans*. In *Flavins and Flavoproteins 1996* (Stevenson, K.J., Massey, V. & Williams, C.H. Jr, eds), pp. 769–772. University of Calgary Press, Calgary.
33. Toomey, D. & Mayhew, S.G. (1998) Purification and characterisation of NADH oxidase from *Thermus aquaticus* YT-1 and evidence that it functions in a peroxide-reduction system. *Eur. J. Biochem.* **251**, 935–945.
34. Koyama, N., Koitabashi, T., Niimura, Y. & Massey, V. (1998) Peroxide reductase activity of NADH dehydrogenase of an alkaliphilic *Bacillus* in the presence of a 22-kDa protein component from *Amphibacillus xylanus*. *Biochem. Biophys. Res. Commun.* **247**, 659–662.
35. Poole, L.B., Higuchi, M., Shimada, M., Li Calzi, M. & Kamio, Y. (2000) *Streptococcus mutans* H_2O_2-forming NADH oxidase is an alkyl hydroperoxide reductase protein. *Free Radic. Biol. Med.* **28**, 108–120.
36. Prongay, A.J., Engelke, D.R. & Williams, C.H. Jr (1989) Characterization of two active site mutations of thioredoxin reductase from *Escherichia coli*. *J. Biol. Chem.* **264**, 2656–2664.
37. Li Calzi, M. & Poole, L.B. (1997) Requirement for the two AhpF cystine disulfide centers in catalysis of peroxide reduction by alkyl hydroperoxide reductase. *Biochemistry* **36**, 13357–13364.
38. Logan, C. & Mayhew, S.G. (2000) Cloning, over-expression and characterization of peroxiredoxin and NADH-peroxiredoxin reductase from *Thermus aquaticus* YT-1. *J. Biol. Chem.*, in press.
39. Zanetti, G., Williams,C.H. Jr & Massey, V. (1968) Influence of photoirradiation on the oxidation–reduction state of thioredoxin reductase. *J. Biol. Chem.* **243**, 4013–4019.
40. Massey, V., Muller, F., Feldberg, R., Schuman, M., Sullivan, P.A., Howell, L.G., Mayhew, S.G., Matthews, R.G. & Foust, G.P. (1969) The reactivity of flavoproteins with sulfite. Possible relevance to the problem of oxygen reactivity. *J. Biol. Chem.* **244**, 3999–4006.
41. Williams, C.H. Jr (1976) Flavin-containing dehydrogenases. In *The Enzymes* (Boyer, P.D., ed.), pp. 89–173. Academic Press, New York.
42. Massey, V. & Palmer, G. (1962) Charge transfer complexes of lipoyl dehydrogenase and free flavins. *J. Biol. Chem.* **237**, 2347–2358.
43. Lennon, B.W. & Williams, C.H. Jr (1996) Enzyme-monitored turnover of *Escherichia coli* thioredoxin reductase: insights for catalysis. *Biochemistry* **35**, 4704–4712.
44. Niimura, Y., Ohnishi, K., Nishiyama, Y., Kawasaki, S., Miyaji, T., Suzuki, H., Nishino, T. & Massey, V. (1997) *Amphibacillus xylanus* NADH oxidase/alkyl hydroperoxide reductase flavoprotein. In *Flavins and Flavoproteins 1996* (Stevenson, K.J., Massey, V. & Williams, C.H. Jr, eds), pp. 741–750. University of Calgary Press, Calgary, Canada.
45. Niimura, Y. & Massey, V. (1996) Reaction mechanism of *Amphibacillus xylanus* NADH oxidase/alkyl hydroperoxide reductase flavoprotein. *J. Biol. Chem.* **271**, 30459–30464.
46. Ohnishi, K., Niimura, Y., Hidaka, M., Masaki, H., Suzuki, H., Uozumi, T. & Nishino, T. (1995) Role of cysteine 337 and cysteine 340 in flavoprotein that functions as NADH oxidase from *Amphibacillus xylanus* studied by site-directed mutagenesis. *J. Biol. Chem.* **270**, 5812–5817.
47. Poole, L.B. (1999) Flavin-linked redox components required for AhpC reduction in alkyl hydroperoxide reductase systems. In *Flavins and Flavoproteins 1999* (Ghisla, S., Kroneck, P., Macheroux, P. & Sund, H., eds), pp. 691–694. Agency for Scientific Publications, Berlin, Germany.
48. Logan, C. & Mayhew, S.G. (1999) Studies on the peroxide-reducing system of *Thermus aquaticus*. In *Flavins and Flavoproteins 1999* (Ghisla, S., Kroneck, P., Macheroux, P. & Sund, H., eds), pp. 401–404. Agency for Scientific Publications, Berlin, Berlin.
49. Kitano, K., Niimura, Y., Nishiyama, Y. & Miki, K. (1999) Stimulation of peroxidase activity by decamerization related to ionic strength: ahpC protein from *Amphibacillus xylanus*. *J. Biochem. (Tokyo)* **126**, 313–319.
50. Wang, P.-F., Veine, D.M., Ahn, S.H. & Williams, C.H. Jr (1996) A stable mixed disulfide between thioredoxin reductase and its substrate, thioredoxin: preparation and characterization. *Biochemistry* **35**, 4812–4819.
51. Veine, D.M., Mulrooney, S.B., Wang, P.-F. & Williams, C.H. Jr (1998) Formation and properties of mixed disulfides between thioredoxin reductase from *Escherichia coli* and thioredoxin: Evidence that cysteine-138 functions to initiate dithiol-disulfide interchange and to accept the reducing equivalent from reduced flavin. *Protein Sci.* **7**, 1441–1450.
52. Niimura, Y., Nishiyama, Y., Takeda, K., Tsuji, H., Ohnishi, K., Watanabe, T., Nishino, T. & Massey, V. (1999) An NADH oxidase functional as alkyl hydroperoxide reductase. In *Flavins and Flavoproteins 1999* (Ghisla, S., Kroneck, P., Macheroux, P. & Sund, H., eds), pp. 677–680. Agency for Scientific Publications, Berlin.
53. Reynolds, C.M. & Poole, L.B. (2000) Attachment of the N-terminal domain of *Salmonella typhimurium* AhpF to *Escherichia coli* thioredoxin reductase confers AhpC reductase activity but does not affect thioredoxin reductase activity. *Biochemistry* **39**, 8859–8869.
54. Williams, C.H. Jr, Arscott, L.D., Muller, S., Lennon, B.W., Ludwig, M.L., Wang, P.-F., Becker, K. & Schirmer, R.H. (2000) Thioredoxin reductase – two modes of catalysis have evolved. *Eur. J. Biochem.* **267**, 6110–6117.
55. Waksman, G., Krishna, T.S.R., Williams, C.H. Jr & Kuriyan, J. (1994) Crystal structure of *Escherichia coli* thioredoxin reductase refined at 2 Å resolution. Implications for a large conformational change during catalysis. *J. Mol. Biol.* **236**, 800–816.
56. Chae, H.Z., Chung, S.J. & Rhee, S.G. (1994) Thioredoxin-dependent peroxide reductase from yeast. *J. Biol. Chem.* **269**, 27670–27678.
57. Nogoceke, E., Gommel, D.U., Kiess, M., Kalisz, H.M. & Flohe, L. (1997) A unique cascade of oxidoreductases catalyses trypanothione-mediated peroxide metabolism in *Crithidia fasciculata*. *Biol. Chem.* **378**, 827–836.
58. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.
59. Smillie, D.A., Hayward, R.S., Suzuki, T., Fujita, N. & Ishihama, A. (1992) Locations of genes encoding alkyl hydroperoxide reductase on the physical map of the *Escherichia coli* K-12 genome. *J. Bacteriol.* **174**, 3826–3827.