Bovine mitochondrial SP-22 is a member of the peroxiredoxin family of peroxidases. It belongs to the peroxiredoxin 2-Cys subgroup containing three cysteines at positions 47, 66, and 168. The cloning and overexpression in *Escherichia coli* of recombinant wild type SP-22 and its three cysteine mutants (C47S, C66S, and C168S) are reported. Purified His-tagged SP-22 was fully active with Cys-47 being confirmed as the catalytic residue. The enzyme forms a stable decameric toroid consisting of five basic dimeric units containing intermolecular disulfide bonds linking the catalytically active Cys-47 of one subunit and Cys-168 of the adjacent monomer. The disulfide bonds are not required for overall structural integrity. The toroidal units have average external and internal diameters of 15 and 7 nm, respectively, and can form stacks in a lateral arrangement of two or three rings. C47S had a pronounced tendency to stack in long tubular structures containing up to 60 rings. Further unusual structural features are the presence of radial spikes projecting from the external surface and ordered electron-dense material within the central cavity of the toroid.

Oxidative stress arises as a consequence of an imbalance in the metabolism of redox-active species. The principal targets of oxidative damage are the key biomolecules of the cell, namely proteins, lipids, and nucleic acids. Accumulation of these damaged biomolecules is contributory to, or characteristic of, the aging process and several major pathologies, particularly neurodegenerative diseases (1, 2). All aerobic organisms generate reactive oxygen species (ROS), as a consequence of normal cellular metabolism, principally via oxidative phosphorylation in the mitochondrion (1, 3). ROS arise as intermediates in the reduction of molecular oxygen and include the superoxide anion, hydrogen peroxide, and the hydroxyl radical generated via the Fenton reaction. Despite the destructive properties of ROS, in several cases physiological concentrations are required for normal cellular function; indeed some such as hydrogen peroxide serve as second messenger molecules in cell signaling pathways (4, 5).

SP-22 is a small (22 kDa) bovine mitochondrial protein, so-called due to its initial identification as a substrate of a mitochondrial ATP-dependent protease (6, 7). It is exclusively located in the matrix of the organelle, constituting ~5% of total matrix protein in bovine adrenal cortex mitochondria. SP-22 is a member of the peroxiredoxin (Prx) family of peroxidase enzymes, possessing the ability to protect several free radical-sensitive enzymes from inactivation by a metal-catalyzed, free radical-generating system (8, 9). Prxs exhibit diverse functions including roles in transcriptional regulation, apoptosis, immunity, and infection; however, the recent major focus of research concerns their antioxidant properties (10, 11).

Prxs can be divided into at least five distinct groups numbered according to their amino acid sequence, tissue distribution, and cellular localization. SP-22 is a 2-Cys Prx containing conserved cysteines at positions 47 and 168 and a third non-conserved cysteine (Cys-66). By amino acid sequence analysis of mature SP-22, Cys-47 was proposed to be the site of catalysis as it contained a sulfenic acid group (12). It was postulated that in the native state of SP-22, the Cys-47 side group forms a sulfenic acid intermediate, enabling it to function as a two-electron redox center.

While both 1- and 2-Cys Prx subgroups contain a basic dimeric unit (13), the 2-Cys group contains intermolecular disulfide bonds that form between the N-terminal residue of one monomer and the C-terminal cysteine of the adjacent subunit as part of the catalytic mechanism (14). The initial stage of catalysis involves the oxidation of the N-terminal cysteines on both subunits to a cysteine-sulfenic acid by hydrogen peroxide or alkyl hydroperoxides. This moiety spontaneously reacts with the C-terminal cysteine of the adjacent subunit to form an intermolecular disulfide bond coupled to the elimination of water (15). In order for the 2-Cys Prx to be converted back to its active state, the disulfide bonds are reduced by thioredoxin or DTT in an artificial assay system. The 2-Cys Prxs are thioredoxin (Trx)-dependent peroxidases as *in vivo* these enzymes utilize Trx exclusively as their electron donor (16). The reduction of the resulting intramolecular disulfide bond formed within Trx is carried out by the cognate NADP-linked flavoprotein thioredoxin reductase.

To date, the oligomeric structures of several Prxs of both the...
1-Cys and 2-Cys sub-groups have been determined successfully. A second toroidal decameric form comprising five dimeric units (α2β5) is an increasingly recurring theme, particularly for the 2-Cys members (17, 18).

Previous published work on SP-22 is extremely limited; therefore, the exact physiological function and biochemical mode of action of the protein remain to be fully elucidated. In this paper we report the overexpression and purification of recombinant SP-22 and analyze its quaternary structure. We highlight some unusual structural features, and we assess the role of the three cysteine residues in its catalytic function and oligomer stability.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of Wild Type and Mutant SP-22**—Precursor SP-22 previously amplified from bovine brain cDNA (Clontech) and cloned into the pCRScript plasmid vector was used as the template for the PCR and catalyzed by Pfu DNA polymerase (Promega). Specific primers used in the amplification reaction are as follows: forward, 5′-TTC CTC TCC TCC CCC CCC CGT CAC CC-3′; and reverse, 5′-CCG CCC GGA TCC TTA GTC ATT TCT CTT C-3′. BamHI recognition sites (indicated in boldface) were incorporated to facilitate the subsequent ligation into the pET14b vector (Novagen). Amplification was carried out in a PTC-100TM programmable thermocycler (MJ Research Inc.). Successful amplification was confirmed by agarose gel electrophoresis.

Site-directed Mutagenesis of SP-22 to convert each of the three cysteines to serine residues was performed using the QuikChange™ Site-directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer’s instructions. Specific oligonucleotide primers were designed, complementary to the region of the gene containing the cysteine residue to be mutated. The annealing temperatures were 45°C (C475S and C666S) and 55°C (C188S).

The fidelity of the amplification reactions was confirmed by DNA sequencing at the Molecular Biology Sequencing Unit, University of Glasgow, Glasgow, Scotland, UK.

*Escherichia coli* DE3 pLysS bacterial cells (Novagen) transformed with the recombinant plasmids were grown at 37°C in Luria broth supplemented with 50 μg/ml ampicillin until an A660 of 0.5 was attained, prior to induction of protein expression by addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Cultures were incubated for a further 5 h at 22°C to promote optimal expression of soluble protein, prior to harvesting the cells by centrifugation at 11,000 × g for 15 min. The bacterial pellet was resuspended in 15 ml of starting buffer (20 mM HEPES buffer, pH 8.0, containing 0.5 M imidazole and 1.0 M NaCl) prior to passage (four times) through a French pressure cell press (Amino) at a pressure of 950 pounds/square inch (6.68 MPa). The mixture was centrifuged (13,800 × g for 15 min) and the supernatant retained. His-tagged enzymes were purified on a metal affinity column (10 × 100 mm, bed volume 7.85 ml), containing SelfPack POROS 20 Metal Chelate Affinity Packing using a BioCAD® 700E Series Perfusion Chromatography® work station (PerSeptive Biosystems), according to the protocol recommended by the manufacturer. Zinc(II) ions were loaded onto the matrix, and recombinant proteins were eluted using an increasing imidazole gradient (0.5 mM to 0.5 M) in the same buffer.

**SDS-PAGE**—Electrophoresis of protein samples was carried out according to the method used by Laemmli (21). For more accurate concentration determination of purified protein, the A280 was measured. The extinction coefficient used for SP-22 and the cysteine mutants was 0.73 (1 mg/ml protein in a cuvette of path length 1 cm), calculated from the amino acid sequence (20).

**SDS-PAGE**—Electrophoresis of protein samples was carried out according to the method used by White et al. (22). Proteins were precipitated with 10% (v/v) trichloroacetic acid and then incubated on ice for 5 min prior to centrifugation at 6,500 × g for 10 min at 4°C. The pellet was resuspended in 600 μl of ice-cold acetone and centrifuged as before. The acetone precipitation step was repeated before final resuspension of the pellet in 30 μl of Laemmli sample buffer (10% (w/v) sucrose, 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.8, and a small amount of pyronin Y dye).

**Circular Dichroism**—Far UV CD spectra over the range 190–260 nm were obtained using a Jasco J-600 spectropolarimeter. Secondary structure estimations were calculated using the SELCON procedure (23).

**Transmission Electron Microscopy**—As an additional purification step prior to TEM, gel exclusion chromatography was performed at room temperature using a pre-packed HiPrep 16/60 Sephacryl S-300 High Resolution column (Amersham Biosciences) connected to a BioCAD® 700E Perfusion Chromatography® work station. The flow rate was 1 ml/min, and the volume of protein loaded was 1 ml (recommended < 2% of bed volume). The column was equilibrated with 2 column volumes of 50 mM KP, buffer 7.0, containing 150 mM NaF and calibrated with a MW-GF-1000 molecular weight marker kit (Sigma) according to the manufacturer’s instructions. SP-22 peak fractions were maintained at 4°C until used.

The single droplet method described by Harris (24) was adopted for the negative staining procedure. Approximately 3 μl of SP-22 (50–100 μg/ml) was loaded onto a carbon-coated electron microscope grid (Agar Scientific, Stansted, UK) for 1 min. Grids were washed with distilled water and stained with a 1% solution of 0.5% (w/v) uranyl acetate, and left to air-dry. Low dose electron micrographs were recorded using a JEOL 1200 EXII transmission electron microscope recording at ×30,000 magnification, ~600–1,000 nm underfocus, onto SO163 film (Eastman Kodak Co.). Two-dimensional averages were calculated using a reference-free alignment algorithm (25). Projected radial density profiles were calculated from top views of SP-22 rings, and their radii were compared by inspection of these plots and the raw images. All image processing was carried out using the SPIDER image-processing package (26).
proteins were present; therefore, no other purification steps were deemed necessary.

**SP-22 Forms a Basic Disulfide-linked Dimeric Unit**—Non-reducing SDS-PAGE analysis of wild type and mutant SP-22s established that SP-22 is isolated in its oxidized form since the protein migrates as a dimeric unit containing two disulfide bonds. Fig. 1 confirms that both Cys-47 and Cys-168 are involved in the formation of these disulfide bonds, since in the absence of DTT the C47S and C168S mutants are in their monomeric form (M1). However, the C66S mutant is dimeric; therefore, Cys-66 does not participate in disulfide interactions. It can be concluded that the disulfide bonds form between the N-terminal cysteine of one monomer and the C-terminal cysteine of the adjacent subunit.

Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid) was used to assess the accessibility of Cys-66 to thiol modification under native and non-native conditions (27). No cysteine residues were accessible in the fully folded wild type SP-22; however, following denaturation in 6 M guanidinium chloride, approximately one (0.75) thiol group (Cys-66) was modified by DTNB. This implies that in the native enzyme Cys-66 is buried within the tertiary structure. To confirm that the accessible residue was Cys-66, the assay was repeated for the C66S mutant. As expected no cysteines were accessible in the C66S mutant in both native and denaturing conditions.

**SP-22 Forms a Larger Oligomeric Structure Corresponding to a Decamer**—SP-22 was shown to adopt a larger oligomeric form using nonspecific cross-linking of purified SP-22 (50 μg) with glutaraldehyde and analysis by non-reducing SDS-PAGE. One major protein band was observed at ~250 kDa, corresponding to a SP-22 decamer (Fig. 2). Four weaker bands were detected at higher mobilities than the decameric band illustrating the intermediate oligomeric states that were found as a result of incomplete cross-linking, corresponding to octamers (~200 kDa), hexamers (~150 kDa), tetramers (~100 kDa), and dimers (~50 kDa) of SP22 subunits. Minor bands corresponding to higher molecular weight species above 500 kDa were also detected. Following independent SDS-PAGE analysis on a 3% (w/v) sodium phosphate gel (Weber-Osborne) (results not shown), it was established that these higher molecular weight species have molecular masses of ~500 and 750 kDa. It is proposed that these bands correspond to double or triple stacks of SP-22 rings, observed frequently in electron microscopy studies (Fig. 4B).

**Secondary Structure Estimation of SP-22**—Far-UV CD measurements over the range 260 to 190 nm were used to estimate the secondary structural composition of SP-22. CD data were analyzed using the SELCON procedure (23) (Table I). It can be seen that the largest secondary structure contribution is attributed to the α-helical content, constituting nearly 50% of the overall secondary structure. β-Sheet content contributes 29.5% of the overall structure.

**Assessment of SP-22 Stability**—The stability of the SP-22 oligomer was assessed by chemical denaturation with urea. SP-22 was unfolded in increasing urea concentrations at room temperature, incubating overnight to allow equilibrium to be reached. Despite this lengthy incubation period and choice of denaturant, complete unfolding, even at 9.5 M urea, was not achieved as judged by far-UV CD, indicating that the native structure of SP-22 was extremely stable.

Between 5 and 6 M urea there is a major unfolding event that is subsequently followed by further gradual unfolding until 9.5 M urea at which stage the oligomer still retains ~25% of its native ellipticity at 222 nm (Fig. 3). These results therefore indicate that unfolding of SP-22 does not follow a simple two-state mechanism (native state → unfolded state) characterized

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**Table I**

| Secondary structure | Proportion present in overall structure |
|---------------------|----------------------------------------|
| α-Helix             | 46.3                                   |
| Anti-parallel β-sheet | 9.1                                   |
| Parallel β-sheet    | 6.6                                    |
| β-Turn              | 13.8                                   |
| Other               | 24.2                                   |

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*L. J. Gourlay and J. G. Lindsay, unpublished results.*
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by a monophasic denaturation curve. The reduced form of SP-22 was slightly less stable, beginning to unfold in 3.75 M urea (Fig. 3). The major loss of structure occurs in the region of 4–5.5 M urea. As for non-reduced wild type SP-22, unfolding is incomplete, and the protein retains −25% native ellipticity in 9.5 M urea.

Unfolding experiments were repeated for the cysteine mutants. The C66S mutant unfolding profile was comparable with that of wild type SP-22, not unexpected given that the disulfide bonds are intact in this mutant. The C47S and C168S mutant SP-22s exhibited overall reduced stability confirming that the disulfide interactions are necessary for structure stability but not oligomer assembly. To prevent the formation of nonspecific disulfide bond formation during unfolding, the experiments with the mutant SP-22s were repeated in the presence of DTT. The effect of DTT on the stability of the mutant SP-22s was more pronounced for C66S and was minor for C47S and C168S, reinforcing the roles of the disulfide bonds in oligomer stability.

Transmission Electron Microscopy—As an extra purification step, wild type SP-22 (purified) was passed down a Sephacryl-300 16/60 HR gel filtration column and, depending on the conditions, eluted as a major peak with an apparent molecular mass of ~450 kDa (fraction B). Occasionally, a minor peak of aggregated material eluting at the void volume was also observed (fraction A). SP-22 was immobilized on a continuous carbon support and stained with 1% (w/v) uranyl acetate. Fraction A showed that SP-22 is toroidal with the presence of long stacks comprising multiple rings aggregated in a lateral fashion, a predominant feature. Analysis of SP-22 from fraction B illustrated a prevalence of single rings although there were significant numbers of double and triple toroids (Fig. 4A). Interestingly, side views were predominantly found in comparison with top views implying some interaction of the protein with the grid.

Image processing was carried out to investigate the ring structure of SP-22 from fraction B at higher resolution. Protein cross-linking experiments indicate that SP-22 is a decameric toroid. Attempts to demonstrate unambiguous 5-fold symmetry by established methods (28) were unsuccessful. Two-dimensional averages of top views calculated by the reference-free alignment method, however, indicate a slightly angular appearance that is consistent with the three-dimensional reconstruction calculated for peroxiredoxin II (29). Radial density profiles were calculated from raw images, and these averages show that SP-22 rings have an internal diameter of ~7 nm and an external diameter of ~15 nm. Another feature of the rings, ubiquitously observed in both raw and two-dimensional averages, was the presence of spikes radiating from the outer edge of the ring. The pronounced modulation of staining repeatedly seen in these images is, we believe, too ordered to be an artifact of the staining procedure. Additionally, ordered electron dense material was observed within the central cavity of the majority of rings. This material appears to be regularly organized, displaying distinct structural features (Fig. 4B).

It appears this central cavity material is probably part of the SP-22 oligomer, as no contaminating E. coli proteins were detected in a sample of purified SP-22 silver-stained following SDS-PAGE. This was also confirmed by immunoblotting with a monoclonal antibody that specifically detects His-tagged proteins. Indeed, silver staining, which can detect 0.1–0.6 ng of protein, and immunoblotting only detected bands corresponding to SP-22, implying the material within the central cavity is part of the overall SP-22 structure and is not contaminating E. coli proteins.

Negative Staining of Reduced Wild Type SP-22—To confirm that the formation of the intermolecular disulfide bonds is not essential for decamer formation, the negative staining procedure was repeated for SP-22 in the presence of 20 mM DTT. Reduced SP-22 remains toroidal confirming that the disulfides are not required for structural integrity. A greater proportion of single rings was observed, however, and stacking was less frequent than for oxidized SP-22. As wild type and mutant SP-22s were generated as His-tagged fusion proteins, it is possible that aggregation may be directed by these His tags being tethered together via interactions with contaminating zinc ions from the purification procedure. This would account for the high proportion of single rings observed in the presence of DTT since this reducing agent binds zinc ions with high affinity (30). Gel filtration studies with thrombin-cleaved SP-22 indicate no significant loss in molecular mass and with the TEM results, suggesting that although the His tags may promote SP-22 to stack, they do not direct it. Interestingly, the apparent molecular mass of SP-22 on gel filtration analysis remained constant at ~450–500 kDa, regardless of the proportion of single toroids observed by TEM. It is suggested that the
Properties of 2-Cys Peroxiredoxin SP-22 Decameric Toroid

high apparent molecular mass relates to the toroidal nature of SP-22, containing a large central cavity that increases its overall effective diameter. Lateral stacks of double and triple toroids of SP-22 have the same effective diameter as single toroids, displaying similar elution characteristics on gel filtration (Fig. 4B).

**Negative Staining of the C47S Mutant of SP-22**—On gel filtration, the C47S mutant showed a single peak at the void volume, corresponding to a molecular mass of at least 2,000 kDa. The structure of this mutant was investigated by negative staining under conditions used for the wild type enzyme. C47S had a tendency to form long stacks of up to 60 rings, similar to those seen in the void volume fraction found in some preparations of wild type SP-22, with single toroids being extremely rare (Fig. 4C). These structures were comparable to protein filaments, stacking in a particularly ordered manner. To determine whether disulfide interactions may mediate stacking, gel filtration was carried out in the presence of DTT (20 mM). The reduced material still eluted at the void volume, confirming that the disulfide bonds are not involved in stacking, and large molecular weight aggregates were still forming. To eliminate the role of the His tags in stacking, gel filtration experiments were carried out in the presence of the chelating agents EDTA and DTT, and by proteolytic removal of the His tags. No reduction in the extent of aggregation was observed with the C47S mutant.

**Peroxidase Activity of SP-22**—SP-22 was confirmed to be active by assessing its ability to protect enolase, a free radical-sensitive enzyme, from inactivation by a metal-catalyzed free radical-generating system (FRS). The activity of enolase was measured by recording the increase in \(A_{340}\), corresponding to the conversion of 2-phosphoglycerate to phosphoenolpyruvate. To assess the protective effect of SP-22, enolase was incubated at 30 °C with the FRS and SP-22. The remaining enolase activity was assessed at regular time intervals. Enolase activity was less than 20% of the controls after 10 min, with ~6% of the original activity being retained after 30 min (Fig. 5). Incubating the FRS with 2 mM EDTA prior to addition of the enolase prevented the inactivation implying that Fe\(^{2+}\) was essential for generating the reactive oxygen species involved in inactivation of enolase. Inactivation did not occur with DTT or FeCl\(_3\) alone. At a ratio of 2:1 (w/w) of SP-22:enolase, protection was ~100% after 20 min. The extent of protection was similar for lower SP-22:enolase ratios; even when the ratio SP-22:enolase was as low as 1:4 (w/w), 70% enolase activity was retained after 20 min. Overall the extent of enolase protection is similar for all SP-22 concentrations, until the lowest proportion of SP-22:enolase (1:8) tested, where the remaining enolase activity was only 31% after 20 min. To ensure protection was specifically due to SP-22 and not the presence of additional protein, controls were performed using bovine serum albumin and catalase. Bovine serum albumin present at maximal levels (66 μg/assay) displayed no protective effect toward enolase. Catalase did protect enolase (Fig. 5), confirming that the hydrogen peroxide generation is necessary for damaging enolase, presumably via its breakdown product, the hydroxyl radical generated via the Fenton reaction.

**Role of the Conserved Cysteines in SP-22 Peroxidase Activity**—To assess the role of the conserved cysteine residues in the catalytic activity of SP-22, the enolase inactivation and protection assays were repeated for the cysteine mutants (Fig. 5). C47S was established to be the catalytic residue, as this mutant displayed no significant protection toward enolase. Less than 10% enolase activity remained following a 20-min incubation period with the FRS. There was no significant difference between the wild type and the C66S and C168S mutants in their protection toward enolase, implying they are not required for Cys-47 to play its role in SP-22 activity. High levels (80 and 75%) of enolase activity remained in the presence of maximal concentrations of C66S and C168S, respectively. It is somewhat surprising that the C168S mutant protects enolase efficiently given its involvement in disulfide bond formation during catalysis. This is unlikely to occur in vivo as thioredoxin reduces the enzyme via an intermediate stage in which it forms a disulfide bond with SP-22. Replacement of Cys-168 by serine would prevent such an interaction. In this artificial assay system, however, DTT may directly reduce the Cys-47 sulfenic acid group back to its cognate thiol without disulfide bond formation. In effect, the C168S mutant protects enolase efficiently given its involvement in disulfide bond formation during catalysis. This is unlikely to occur in vivo as thioredoxin reduces the enzyme via an intermediate stage in which it forms a disulfide bond with SP-22. Replacement of Cys-168 by serine would prevent such an interaction. In this artificial assay system, however, DTT may directly reduce the Cys-47 sulfenic acid group back to its cognate thiol without disulfide bond formation. In effect, the C168S mutant of SP-22 is acting as a 1-Cys peroxiredoxin under these conditions.

**DISCUSSION**

Several enzymatic defense systems have evolved to protect the cell from oxidative stress including the superoxide dis-
mutase, catalase, glutathione peroxidase, thioredoxin, and peroxiredoxin systems. With respect to peroxidase systems within the mitochondrion, glutathione peroxidase activity is evident; however, catalase activity has only been detected in mitochondria isolated from rat heart (31). Therefore, it is likely that SP-22 in conjunction with the thioredoxin system forms an important defense against oxidative modification of mitochondrial DNA and proteins. Such modifications are characteristic of aging and several pathologies due to the vital metabolic roles played by these molecules (32).

Research into the precise physiological role of SP-22 has been limited, with studies focusing on its ability to protect several free radical-sensitive enzymes (8, 9). In addition to SP-22, only one other mitochondrial Prx (PRDX5) has been identified. PRDX5 is a 2-Cys member; however, it represents a novel subgroup of Prxs as it does not contain intermolecular disulfides and is, in fact, exclusively monomeric (33). Although PRDX5 contains three cysteine residues, only the N-terminal residue is conserved with the other 2-Cys members. In general, the 2-Cys Prxs studied to date (excluding PRDX5) all exist as homodimeric enzymes that further associate in some case to give a larger oligomeric form. This larger assembly is toroidal in conformation, formed by the interaction of five dimers joined end-to-end (17, 19). AhpC isolated from Mycobacterium tuberculosis and the human erythrocyte protein TPx-B have been demonstrated to undergo a transition between dimeric and decameric forms according to ionic strength and pH, respectively (34).

Following our successful overexpression of recombinant SP-22 as a His-tagged protein in bacterial cells, and from the biophysical analyses, it is clear that SP-22 is distinct in structure from PRDX5 and instead conforms to typical 2-Cys Prxs, forming a toroidal decamer composed of a basic disulfide-linked dimeric unit, extremely resistant to chemical denaturation. The activity of SP-22 is reliant on intermolecular disulfide bond formation via the catalytic Cys-47 residue and Cys-168, as has been established by assessing enolase protection against inactivation by an in vitro metal-free radical-generating system. This non-equivalence of Cys-47 and Cys-168 was supported in vitro with E3, both being members of the pyridine nucleotide-disulfide family of oxidoreductases. Furthermore, in M. tuberculosis,

It is apparent that recombinant SP-22 in accordance with findings for the isolated native enzyme does not exist as a single species (35) and can form lateral stacks consisting of two and three rings. Increased stacking was observed for the C47S mutant, with the association of more than 60 rings. This phenomenon has also been reported for the human erythrocyte protein TPx-B (29); however, stacking was more prevalent for recombinant SP-22. Novel features, which have not been reported to date for other peroxiredoxins, are as follows: (a) radial spokes projecting outwards from the central cavity of the decamer, and (b) material within the central cavity. These findings require further exploration.

Elucidating the function of SP-22 is also of particular importance, not only to its intracellular location but due to a recent observation that SP-22 co-elutes with the dihydrolipoamide dehydrogenase (E3) component of the pyruvate dehydrogenase complex upon separation of the complex into its enzyme components (E1, E2, and E3) by size exclusion chromatography. Further interaction studies using isothermal titration calorimetry and surface plasmon resonance indicate a binding affinity between E3 and SP-22 in the micromolar range. The functional significance of such an interaction requires further investigation. All the enzyme components of the 2-oxoacid dehydrogenase possess sulfur-containing moieties, which are potential targets for oxidative damage and may induce further damage via generation of reactive sulfur species. It has been reported that E3 has NADH oxidase activity and can generate hydrogen peroxide and, to a lesser extent, the superoxide anion via the reduction of molecular oxygen using reducing equivalents from NADH (36). This may account for the direct physical interaction between E3 and SP-22, allowing the latter enzyme to scavenge hydrogen peroxide generated in the local environment.

A connection between SP-22 and E3 is indicated by amino acid sequence comparisons alone. SP-22 has high amino acid sequence identity with the Salmonella typhimurium 2-Cys Prx AhpC. During catalysis AhpC is reduced by its cognate flavoprotein AhpF, which has high amino acid sequence identity with E3, both being members of the pyridine nucleotide-disulfide family of oxidoreductases. Furthermore, in M. tuberculosis,
AhpC catalyzes the removal of hydroperoxides using a system that lacks AhpF. Instead, electrons are transferred from NADH to E3, succinyl acetyltransferase (E2), and an adaptor protein designated as AhpD, before finally reducing AhpC (37). AhpD contains an active site sequence conserved with Trx. Therefore, an alternative, indirect functional relationship may exist between E3 and SP-22 via an interaction with the thioredoxin system. Indeed, Trx is reported to activate the 2-oxoacid dehydrogenase complex, and indeed other mitochondrial enzymes, by forming specific protein-protein interactions, suggesting a direct involvement in their regulation (38). It is suggested that Trx is competitive with respect to E3 in the oxidation of the dihydrolipoamide intermediate and can also oxidize the E2 component. Free lipoic acid has further been shown to reduce SP-22, implying an alternative means of interacting with the 2-oxoacid dehydrogenase complexes via E2.

The modification and loss of activity of the 2-oxoacid dehydrogenase complexes, particularly the 2-oxoglutarate dehydrogenase complex, and indeed other mitochondrial enzymes, is frequently observed in oxidative stress-related disorders and is responsible for the large decrease in glucose metabolism in the brains of these patients (39). The interaction of SP-22 with the 2-oxoacid dehydrogenase complexes may therefore serve to protect them from reactive oxygen species generated either via the electron transport chain or directly via the intrinsic NADH oxidase activity of E3.

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