Protein Science (1998), 7:2465–2468. Cambridge University Press. Printed in the USA.
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FOR THE RECORD

Evidence that peroxiredoxins are novel members of the thioredoxin fold superfamily

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(RECEIVED April 10, 1998; ACCEPTED July 13, 1998)

Abstract: Peroxiredoxins catalyze reduction of hydrogen peroxide or alkyl peroxide, to water or the corresponding alcohol. Detailed analysis of their sequences indicates that these enzymes possess a thioredoxin (Trx)-like fold and consequently are homologues of both thioredoxin and glutathione peroxidase (GPx). Sequence- and structure-based multiple sequence alignments indicate that the peroxiredoxin active site cysteine and GPx active site selenocysteine are structurally equivalent. Homologous peroxiredoxin and GPx enzymes are predicted to catalyze equivalent reactions via similar reaction intermediates.

Keywords: cysteine sulfenic acid; glutathione peroxidase; oxidative stress; reactive oxygen species; thiol specific antioxidant

Reactive oxygen species (ROS) are generated as by-products of normal biochemical processes, as weapons against infection and, at lower levels, as secondary messengers during cell signaling (Halliwell & Gutteridge, 1989; Pahl & Baeuerle, 1994). Oxidative stress is caused by the buildup of ROS within tissues and causes damage to proteins, nucleic acids, and lipids (Halliwell & Gutteridge, 1989). Cellular life has evolved a range of ROS-reducing enzymes that regulate the levels of ROS within tissues. These enzymes include catalase, glutathione peroxidase (GPx), and an emerging homologous family of antioxidant enzymes termed the peroxiredoxins (Chae et al., 1994b).

The peroxiredoxin (Prx) family, previously referred to as the thiol-specific antioxidant (TSA)/alkyl hydroperoxide reductase C (AhpC) family, includes bacterial AhpC proteins (Jacobson et al., 1989) and eukaryotic thioredoxin peroxidases (TPxs) (Chae et al., 1994a). In common with GPxs, Prxs are able to reduce hydrogen peroxide and alkyl hydroperoxides (of the form ROOH) to water or the corresponding alcohol (ROH), respectively (Jacobson et al., 1989; Lim et al., 1993; Chae et al., 1994a; Cha & Kim, 1995; Tsuji et al., 1995; Poole and Ellis, 1996; Bruchhaus et al., 1997; Jin et al., 1997; Nogoceke et al., 1997; Shau et al., 1997; Kang et al., 1998b). However, they appear to achieve these similar results by different means. GPxs are selenoproteins that utilize an active site selenocysteine residue in the hydroperoxide reduction cycle (Ursini et al., 1995), whereas the activity of the Prxs is dependent upon a single conserved cysteine that is essential for the hydroperoxide reduction step (Chae et al., 1994c). There is no evidence that Prxs utilize or contain any metal ions, prosthetic groups, or cofactors.

Crystal structures reported for the selenium-dependent GPx (Ladenstein et al., 1979; Epp et al., 1983; Ren et al., 1997) show that these enzymes represent a subclass of a presumed homologous superfamily of enzymes that each contain a thioredoxin-like fold and each interact with either thiol- or disulfide-containing substrates (Martin, 1995). These enzymes may be divided into five sequence and structural subclasses: (1) GPx, (2) glutathione S-transferase (GST), (3) thioredoxin (TRx), (4) glutaredoxin (GRx), and (5) DsbA, that catalyzes disulfide formation in vivo. The latter three enzymes contain active-site CXXC motifs and their activities depend on the lowered pK_a of the N-terminal cysteine residue, the identity of the intervening "XX" residues, and the effect of the dipole moment of a neighboring α-helix (Holmgren, 1995; Chivers et al., 1997); the selenocysteine of GPx is structurally coincident with the Trx N-terminal cysteine (Ladenstein et al., 1979; Epp et al., 1983; Ren et al., 1997).

By contrast to the GPxs, neither the structure nor the detailed catalytic mechanism of the Prxs is understood, although a mechanism has been proposed (Chae et al., 1994a; Poole, 1996; Kang et al., 1998a). What is known is that the antioxidant activity is dependent upon the presence of a conserved cysteine in either one (for the "1-Cys Prxs") or both (for the "2-Cys Prxs") of a pair of conserved motifs (Chae et al., 1994b): the N-terminal cysteine is absolutely conserved, which is consistent with its essential role in catalysis (Chae et al., 1994c; Tsuji et al., 1995). For the 2-Cys Prxs, the N-terminal conserved cysteine is believed to be oxidized to sulfenic acid during peroxide reduction, and in eukaryotes regeneration of the enzyme in vivo is achieved via a sulfhydryl-reducing enzyme system composed of NADPH, Trx, and Trx reductase (Chae et al., 1994a; Cha & Kim, 1995; Poole, 1996) (consequently many of the eukaryotic 2-Cys Prxs are known as thioredoxin peroxidases). The C-terminal cysteines of 2-Cys TPxs are proposed to be involved in their regulation by Trx (Chae et al., 1994b). The in

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vivo activities of bacterial 2-Cys TPxs are maintained by a 52 kDa Trx reductase-like flavoprotein that transfers electrons from NADH or NADPH (Jacobson et al., 1989; Tartaglia et al., 1990; Poole, 1996). Regeneration of the in vivo activities of oxidized 1-Cys TPxs is less clear, although it is likely to depend upon small thiol reducing agents such as cysteine or thioglycerol (Chae et al., 1994a) but not apparently glutathione (Kang et al., 1998a).

Results and discussion: *Sequence analysis:* We have attempted to improve on previous attempts to predict the remote ancestry of Prxs (Chae et al., 1994b; Hudson-Taylor et al., 1995; Montemartini et al., 1998; Schröder et al., 1998). Position-specific iterative BLAST (PSI-BLAST) (Altschul et al., 1997) database searches (using $E < 0.001$) strongly suggest that Prxs represent a hitherto unknown sixth subclass of the thioredoxin fold-containing enzyme superfamily (for details see Fig. 1 legend).

Optimum alignment of Prx sequences to other thioredoxin-homologous sequences was problematic given that the thioredoxin fold is susceptible to insertions of secondary structures at the N- and C-termini, and/or within polypeptides linking secondary struc-

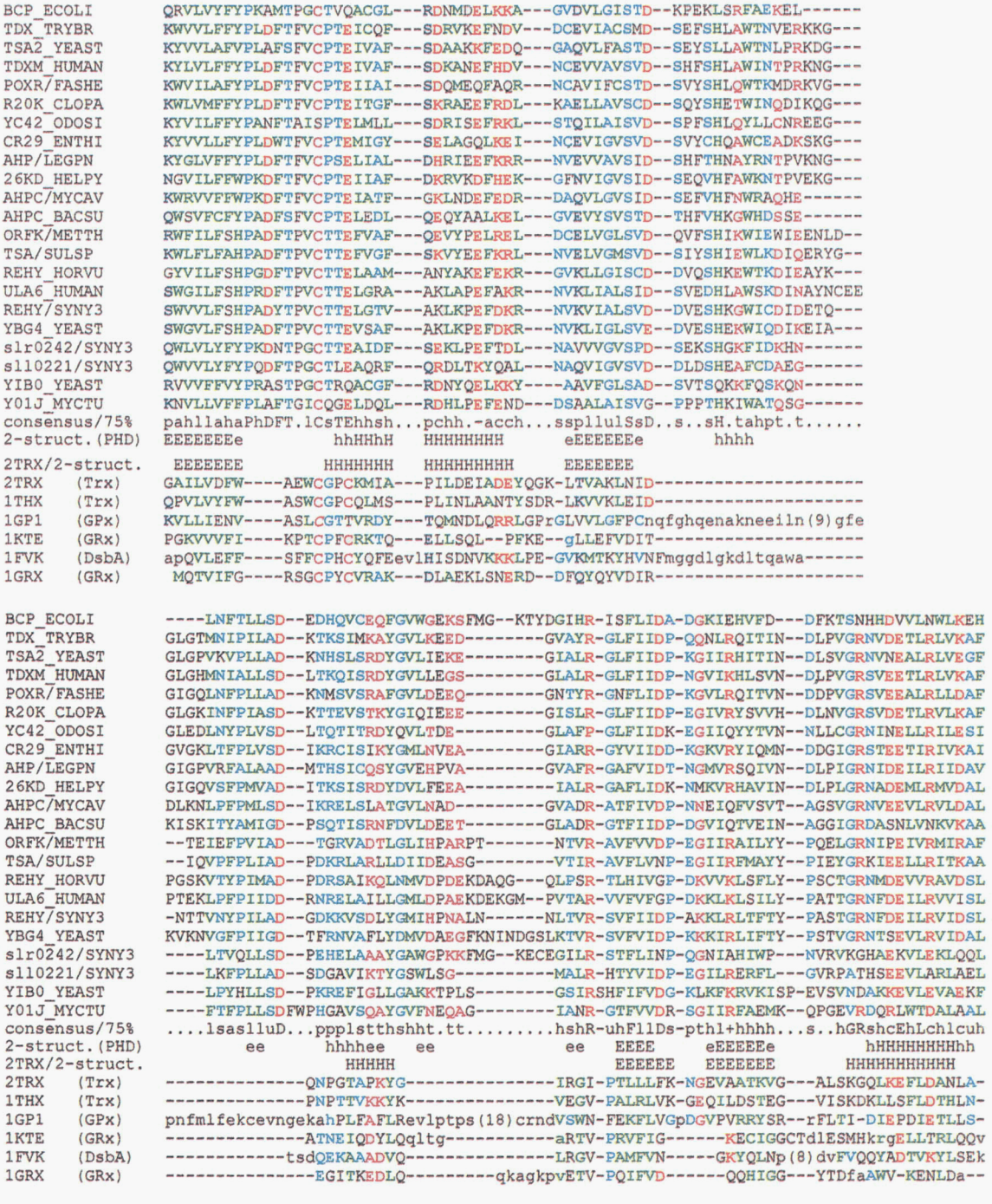

Fig. 1. *See caption on facing page.*

tures $\beta2$–$\alpha2$, $\alpha2$–$\beta3$, and $\beta4$–$\alpha3$ (Martin, 1995). Alignment was guided by: (1) CLUSTAL-W-derived alignments (Thompson et al., 1994), (2) the predicted secondary structures of previously known Prx homologues (Rost & Sander, 1993), and (3) a multiple alignment derived from an automatically-derived structural alignment of known Trx-like structures (Epp et al., 1983; Katti et al., 1990, 1995; Sodano et al., 1991; Martin et al., 1993; Saarinen et al., 1995) using Dali (Holm & Sander, 1998). The final alignment (Fig. 1) predicts N- and C-terminal extensions to thioredoxin-like folds in Prxs, and 21–30 residue insertions within polypeptides linking $\beta2$ and $\alpha2$ (Fig. 2). The equivalent position in the DsbA structure accommodates a large 65 residue helical domain (Martin et al., 1993).

Active site: Superposition of predicted Prx and known Trx secondary structures indicate that the Cys that is essential for catalysis (Chae et al., 1994c; Tsuji et al., 1995) corresponds with both the N-terminal active site Cys32 of Trx and the catalytic selenocysteine of GPx (Fig. 1). This alignment, rather than an alternative that aligns the Prx active cysteine with Cys35 of Trx, is preferred since both the conserved active cysteine of Prxs and Cys32 of Trxs are known to be solvent-exposed whereas Trx Cys35 is more buried (Holmgren, 1995; Kang et al., 1998a).

The alignment represented in Figure 1 predicts the TPx active site cysteine to be positioned at the N-terminus of a long α-helix following a β-strand, as predicted previously (Baier & Dietz, 1996). By analogy to the results of structural and theoretical studies (Holmgren, 1995; Kortemme & Creighton, 1995), it is predicted that the pK_a of the Prx active site cysteine is substantially lowered particularly given that Prx enzymes often contain an active site Cys-Pro dipeptide (Kortemme & Creighton, 1995) (Fig. 1).

Catalytic mechanisms of Prx and Gpx enzymes involve similar oxidation products: The original proposal for the Prx enzyme mechanism (Chae et al., 1994a) remains plausible for all members of the family. This is despite differences in the manner by which the reduced forms of the 1-Cys and 2-Cys enzymes are regenerated (Poole, 1996; Kang et al., 1998a). A central feature of the model is oxidation of the conserved N-terminal Cys to Cys-SOH sulfenic acid with simultaneous reduction of peroxide to water or alcohol. This is consistent with the crystal structure of a 1-Cys Prx (Kang et al., 1998a; H.J. Choi, S.W. Wang, C.H. Yang, S.G. Rhee, S.E. Ryu, unpubl. obs.) described as containing a cysteine sulfenic acid within the active site. Formation of sulfenic acid intermediates is well characterized for a range of peroxidases (Claiborne et al., 1993) that include selenium-dependent GPx (Epp et al., 1983). It is inferred that the Prx and GPx enzyme families possess common folds and employ similar catalytic mechanisms.

Regeneration of oxidized enzymes: Analysis of the sequence of a reported nonselenium dependent GPx (Shichi & Demar, 1990; Frank et al., 1997; Munz et al., 1997) clearly identifies it as a 1-Cys Prx (this work) indicating that regeneration of the oxidized form of some 1-Cys Prx enzymes is glutathione-dependent (Shichi & Demar, 1990; Frank et al., 1997; Munz et al., 1997) although this does not appear to be the case for all 1-Cys Prx enzymes (Kang et al., 1998a).

Regeneration of oxidized AhpC, a 2-Cys Prx, is mediated by AhpF, a thioredoxin reductase homologue (Tartaglia et al., 1990). Thus one homologous family, the thioredoxin reductases, acts to reduce another, consisting of Trx-like Prx enzymes. It appears that not only have homologous families of Trx-like and Prx-like enzymes been preserved throughout evolution, but so have their mechanisms for regeneration of their oxidized forms.

Conclusions: The peroxiredoxin family of enzymes is predicted to contain a domain with a thioredoxin-like fold and with inser-

Fig. 1. (*facing page*) Multiple alignment of known Prx homologues (Chae et al., 1994b; Hudson-Taylor et al., 1995; Montemartini et al., 1998) with a structure-based alignment (Holm & Sander, 1998) of *Escherichia coli* thioredoxin [PDB code: 2TRX] (Katti et al., 1990), *Anabaena* sp. thioredoxin-2 [1THX] (Saarinen et al., 1995), bovine glutathione peroxidase [1GP1] (Epp et al., 1983), pig liver glutaredoxin [1KTE] (Katti et al., 1995), *E. coli* DsbA [1FVK] (Martin et al., 1993), and *E. coli* glutaredoxin [1GRX] (Sodano et al., 1991) sequences. Residues are colored according to a 75% consensus of the Prx sequences: aliphatic [l: ILV] or aromatic [a: FWHY] or hydrophobic [h: ACFGHIKLMRTVWY] in green; charged [c: DEHKR] or negatively charged [DE] or positively charged [HKR] in red; polar [p: CDEHKNQRST] or small [s: ACDGNPSTV] or tiny [u: AGS] in cyan; and turn-like [t: ACDEGHKNQRST] in magenta. Sequences of known structures (2TRX, 1THX, 1GP1, 1KTE, 1FVK, and 1GRX) are colored according to the Prx 75% consensus except for the active site cysteines, colored in magenta (the selenocysteine in 1GP1 is represented by "C" in italics). The YC42 ODOSI sequence lacks a predicted active site cysteine residue. Uppercase characters in the structure-based alignment represent residues structurally equivalent with 1THX, whereas lowercase characters represent those that are nonequivalent with 1THX. Numbers in parentheses represent residues excised from the alignment. Predicted (Rost & Sander, 1993) secondary structure of Prxs (E/e represents a β-strand, whereas H/h represents an α-helix; predicted accuracy 72% [lower case]/82% [upper case]) is given below the Prx alignment and above the known secondary structure of *E. coli* thioredoxin (Katti et al., 1990). Sequence analysis of Prx homologues used the PSI-BLAST algorithm (Altschul et al., 1997). For example, using mouse MER5 as the query sequence, a dsbA homologue (GenBank identifier [gi] 2634185) was detected by pass 2, thioredoxin homologues (gi 2633755 and 312981) by pass 3, and a GPx homologue (gi 2632109) was detected by pass 5. In addition, a gapped BLAST search using the *Bacillus subtilis* resA sequence yielded significant similarities to dsbA and thioredoxin-like enzymes ($E < 1 \times 10^{-8}$) as well as the known (Hudson-Taylor et al., 1995) Prx-like family member, bacterioferritin comigratory protein (bcp) from *Mycobacterium tuberculosis* ($E = 8 \times 10^{-7}$). Similar results were obtained using Ssearch (Pearson, 1991): for example, using *B. subtilis* resA as a query sequence yielded significant similarities with both a dsbE-like enzyme from *Bradyrhizobium japonicum* (tlpA; $E = 1.6 \times 10^{-9}$) and the Prx-like bcp from *M. tuberculosis* ($E = 1.7 \times 10^{-6}$). SwissProt codes (beginning with either P or Q) or GenBank identifier codes, and residue limits for these sequences are, from the top: P23480 (31–155); Q26695 (37–163); Q04120 (33–159); P30048 (93–219); 1850611 (32–158); P23161 (35–161); P49537 (36–161); P19476 (72–198); 965473 (36–162); P21762 (34–159); 388902 (46–168); P80239 (32–155); 620126 (29–154); 1045502 (30–155); P52572 (31–162); P30041 (32–167); 1652457 (30–157); P34227 (76–210); 1651777 (32–156); 1651799 (66–182); P40553 (92–212); and, Q10520 (30–151). Species: BACSU, *B. subtilis*; CLOPA, *Clostridium pasteurianum*; ECOLI, *E. coli*; ENTHI, *Entamoeba histolytica*; FASHE, *Fasciola hepatica*; HELPY, *Helicobacter pylori*; HORVU, *Hordeum vulgare* (barley); HUMAN, *Homo sapiens*; LEGPN, *Legionella pneumophila*; METTH, *Methanobacterium thermoautotrophicum*; MYCAV, *Mycobacterium avium*; MYCTU, *Mycobacterium tuberculosis*; ODOSI, *Odontella sinensis*; SULSP, *Sulfolobus* sp.; SYNY3, *Synechocystis* sp. PCC6803; TRYBR, *Trypanosoma brucei*; and YEAST, *Saccharomyces cerevisiae*.

tions preceding, succeeding, and intervening within the domain. The Prx active site cysteine is predicted to be structurally equivalent to the active site selenocysteine of GPx enzymes. Prx and GPx enzymes employ analogous (seleno)cysteine sulfenic acid intermediates during their enzymatic reactions. It is plausible that, by analogy to their established ability to reduce peroxides of the form ROOH → ROH, Prxs may also be able to catalyze the reduction within a protein substrate of a cysteine sulfenic acid group of the form RSOH → RSH, thereby providing a direct means of redox regulation.

Note added in proof: Following the submission of this manuscript, a description of the crystal structure of a human Prx enzyme was published (Choi H-J, Kang SW, Yang C-H, Rhee SG, Ryu S-E. 1998. Crystal structure of a novel human peroxidase enzyme at 2.0 Å resolution. *Nature Struct Biol 5*:400–406). Predictions of the Prx fold, secondary structures, and active site are in accordance with this crystal structure.

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