A novel peroxiredoxin of the plant *Sedum lineare* is a homologue of *Escherichia coli* bacterioferritin co-migratory protein (Bcp)

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We cloned a gene encoding a 17-kDa protein from a cDNA library of the plant *Sedum lineare* and found that its deduced amino acid sequence showed similarities to those of *Escherichia coli* bacterioferritin co-migratory protein (Bcp) and its homologues, which comprise a discrete group associated with the peroxiredoxin (Prx) family. Studies of the recombinant 17-kDa protein produced in *E. coli* cells revealed that it actually had a thioredoxin-dependent peroxidase activity, the hallmark of the Prx family. PrxQ, as we now designate the 17-kDa protein, had two cysteine residues (Cys-44 and Cys-49) well conserved among proteins of the Bcp group. These two cysteines were demonstrated

to be essential for the thioredoxin-dependent peroxidase activity by analysis of mutant proteins, suggesting that these residues are involved in the formation of an intramolecular disulphide bond as an intermediate in the reaction cycle. Expression of PrxQ suppressed the hypersensitivity of an *E. coli* *bcp* mutant to peroxides, indicating that it might exert an antioxidant activity *in vivo*.

Key words: *bcp* gene, thiol redox system, thioredoxin-dependent peroxidase.

INTRODUCTION

Organisms living or transiently staying in aerobic environments must cope with reactive oxygen species, most notably hydroxyl radicals, that arise mainly from the incomplete reduction of molecular oxygen [1]. Reactive oxygen species as a whole have the potential to damage all cellular constituents, but lesions in DNA and membranes are of critical importance for the cell. Lipid peroxidation can generate highly toxic reactive species such as alkoxyl and peroxy radicals, and lipid hydroperoxide, within the membrane [2,3]. To counter these detrimental effects caused by reactive oxygen species, organisms have evolved an array of defence mechanisms that prevent or repair oxidative damage [4,5]. For prevention of such damage, a number of antioxidant proteins that have the ability to reduce H₂O₂ and alkyl hydroperoxides are known [5]. The most recently discovered among these are peroxidases that reduce hydroperoxides with electrons derived from NADPH through the thioredoxin (Trx)/Trx reductase pathway or from some unknown reducing agent(s) [6–9]. These peroxidases are well conserved from prokaryotes to eukaryotes and form a large group now called the peroxiredoxin (Prx) family, or the AhpC (22-kDa subunit of *Escherichia coli* alkyl hydroperoxide reductase)/TSA family [10].

Bacterioferritin co-migratory protein (Bcp) was found initially in *E. coli* as a protein with a molecular mass of 18 kDa which co-migrated with bacterioferritin in SDS/PAGE [11]. A gene (*bcp*) encoding Bcp is located at 53 min in the genetic map of *E. coli* K12 [12]. Analysis of the nucleotide sequence of *bcp* revealed that its deduced amino acid sequence has similarities with those of the

Prx proteins [7]. A number of *bcp* homologues have been found in genomic sequences of prokaryotes and lower eukaryotes, forming a discrete cluster associated with the Prx family [10]. Very recently, *E. coli* Bcp has been found to have a Trx-dependent peroxidase activity [13].

In this study, we found a novel Prx protein, peroxiredoxin Q (PrxQ), from the flowering plant *Sedum lineare*, which exhibited a Trx-dependent peroxidase activity. It is the first member in the Bcp cluster of the Prx family to be found in higher eukaryotes.

EXPERIMENTAL

Organisms, plasmids, phages and media

The plant *S. lineare*, a member of the subfamily Sedoideae of the family Crassulaceae, was obtained in the city of Fukuoka, Japan, grown in our laboratory and used before bearing flowers. Products of PCR were cloned into pMOSBlue (Pharmacia) or pCR2.1 (Invitrogen), and introduced into *E. coli* DH5 α [14]. Plasmid pET11a (Novagen) was used to express a recombinant protein in *E. coli* BL21 DE3 [15]. cDNA was cloned into λ gt11 (Stratagene). pUC18 [16] was used for routine recloning and subcloning. Luria broth and Luria agar (Luria broth solidified with 1.5% agar) were used for growth of *E. coli* cells.

Protein purification and amino acid sequence analysis

Purification of a 17-kDa protein from *S. lineare* was carried out as follows. About 1 kg of *S. lineare* stems and leaves was frozen

Abbreviations used: Trx, thioredoxin; Prx, peroxiredoxin; Bcp, bacterioferritin co-migratory protein; PrxQ, peroxiredoxin Q; IPTG, isopropyl β -D-thiogalactoside; MALDI-TOF, matrix-associated laser-desorption ionization–time-of-flight; t-BOOH, t-butyl hydroperoxide; CM-OOH, cumene hydroperoxide; AMS, 4-acetamido-4'-maleimidyl-stilbene-2,2'-disulphonate; AhpC, 22-kDa subunit of *Escherichia coli* alkyl hydroperoxide reductase; TPx, thiol peroxidase.

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at -80°C , immersed in 1.5 l of an extraction buffer [50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 2 mM PMSF and 10% glycerol], and homogenized in a blender in a batchwise manner. The homogenate was filtered through four layers of gauze, followed by centrifugation at 11 000 *g* for 30 min, and the filtrate was mixed with twice the volume of ice-cold ethanol and centrifuged at 10 000 *g* for 15 min. The precipitate was suspended in 20 ml of the extraction buffer and applied to a gel-filtration column (Sephacryl S-200, Pharmacia; 1.7 cm $\times$ 116 cm). Fractions containing proteins with molecular masses of $\approx$ 17 kDa were pooled and loaded on to a phosphocellulose column (P11, Whatman; 2 cm $\times$ 25 cm) equilibrated with the extraction buffer, which was then eluted with 240 ml of a linear NaCl gradient from 0 to 1.0 M in the extraction buffer. A fraction that was eluted at $\approx$ 0.5 M NaCl was subjected to SDS/PAGE, and proteins on the gel were transferred to a poly(vinylidene fluoride) membrane. After being stained with Coomassie Brilliant Blue R250, a major protein band with a molecular mass of $\approx$ 17 kDa was cut out and analysed in an automatic protein sequencer (Applied Biosystems model 476A, Perkin-Elmer Cetus) for the N-terminal amino acid sequence.

Purification of the recombinant 17-kDa protein was performed as follows. *E. coli* BL21 DE3 harbouring the appropriate recombinant plasmids was grown to a D_{540} value of 0.5 in 300 ml of Luria broth containing 50 $\mu\text{g}/\text{ml}$ ampicillin. Isopropyl β -D-thiogalactoside (IPTG, 1 mM) was then added to the culture, which was incubated for another 2 h to overproduce the recombinant proteins. The cells were collected by centrifugation at 9000 *g* for 10 min, resuspended in 10 ml of TE buffer [50 mM Tris/HCl (pH 7.5)/1 mM EDTA], and disrupted by sonic oscillation (twice for 5 min each) with a sonicator (UR-150P, Tominaga Works). After centrifugation at 10 000 *g* for 15 min, the supernatant was saved. Ammonium sulphate was added to this at 55% saturation, and the precipitate was dissolved in 3 ml of TE buffer. After centrifugation to remove insoluble materials, the solution was dialysed against TE buffer with a dialysis cassette (Slide-A-Lyser, Pierce) for 24 h, and applied to an anion-exchange column (Poros HQ/M, PerSeptive) equilibrated with TE buffer, which was eluted with 26 ml of a linear NaCl gradient from 0 to 1.0 M in TE buffer. The 17-kDa proteins were usually eluted at 40 mM NaCl. The final preparation was analysed by SDS/PAGE for purity, and protein concentration was determined by using a BCA (bicinchoninic acid) Protein Assay kit (Pierce).

Construction of an *S. lineare* cDNA library

Total RNA was purified from *S. lineare* using an RNA-extraction kit (RNeasy Plant Mini Kit, Qiagen), and cDNA was synthesized using the cDNA Synthesis Kit (Pharmacia). The cDNA was cloned into $\lambda\text{gt}11$ with $\lambda\text{gt}11/\text{EcoRI}/\text{CIAP}$ (calf intestine alkaline phosphatase)-treated Vector Kit (Stratagene). All procedures were done according to the manufacturers' instructions.

Plaque hybridization

Plaque hybridization was achieved by a standard method [14]. Oligonucleotides (5'-CCTACTTTAAAGATCAAGA-3' and 5'-CCTACTCTTAAAGATCAAGA-3') expected to hybridize to the N-terminal sequence of the *S. lineare* 17-kDa protein were used as probes for plaque hybridization.

Construction of *E. coli* strains overexpressing the 17-kDa protein

For construction of an *E. coli* strain overexpressing the 17-kDa protein, the nucleotide sequence encoding the mature 17-kDa

protein was amplified from the DNA of a recombinant phage ($\lambda\text{gt}11\text{SLQ1}$) by PCR with oligonucleotide primers (upper primer, 5'-GGGCTAGCAAGGTTACGAAAGGGTCGACGC-3'; lower primer, 5'-CTCGCTAGCATGAAACAAACAACCTAAGC-3'). The PCR product was cloned into pMOS-Blue by the TA cloning method, and further subcloned into pET11a at the *NheI* site by making use of the same restriction sites present near both ends of the PCR product, resulting in pKD601. *E. coli* BL21 DE3 harbouring pKD601 produced, by inference, a recombinant 17-kDa protein with three additional amino acid residues (Met-Ala-Ser) at the N-terminus of the mature 17-kDa protein.

Site-directed mutagenesis

pKD603 and pKD604, derivatives of pKD601, expressing mutant 17-kDa proteins (C44 $\rightarrow$ S and C49 $\rightarrow$ S, respectively) were constructed using the unique-site elimination (U.S.E.) method [17] with the U.S.E. Mutagenesis Kit (Pharmacia) and single-stranded oligonucleotides containing 1-bp mismatches that would generate the desired mutations. Briefly, a two-oligonucleotide system was used, in which one oligonucleotide named the target mutagenic primer introduced the desired mutation into a known sequence of the plasmid DNA while the other oligonucleotide, named the U.S.E. selection primer, changed a unique non-essential restriction site in the plasmid for the purpose of selection of mutated plasmids. The oligonucleotides 5'-pd(CATGCCTGCTTGGTGGATCCTGGAGTTTCATC)-3' and 5'-pd(CGAATCTCTGAATGCAGATGCCTGCTTGGTGC)-3' (where pd stands for phosphorylated deoxy) were used as target mutagenic primers for construction of pKD603 and pKD604, respectively, whereas the oligonucleotide 5'-pd(CTCTTCCTTTTTCAGGCCTATTGAAGCATTATCAGG)-3' was used as the U.S.E. selection primer to convert the *SspI* site into a *StuI* site.

DNA sequencing

DNA sequencing was carried out by using a dideoxy sequencing kit (AutoRead Sequencing kit, Pharmacia) with plasmid templates and an automated DNA sequencer (ALF DNA sequencer II, Pharmacia).

Matrix-associated laser-desorption ionization-time-of-flight (MALDI-TOF) MS

A solution of the test protein (1.5 $\mu\text{g}/\mu\text{l}$ in TE buffer) was diluted 10-fold with a 2:1 0.1% trifluoroacetic acid/acetonitrile solvent mixture (TA solution). Then 2 μl of the dilution was mixed with 3 μl of saturated sinapic acid in TA solution, and 1 μl of this mixture was loaded on to the sample pin of the MALDI-TOF mass spectrometer (ProteinTOF, Bruker).

Peroxidase assay

Trx-dependent peroxidase activity was measured indirectly by oxidation of NADPH [8]. The complete reaction mixture (500 μl) contained 50 mM Hepes/NaOH (pH 7.0), 40 $\mu\text{g}/\text{ml}$ *E. coli* Trx (Wako Chemicals, Osaka, Japan), 12.5 $\mu\text{g}/\text{ml}$ *E. coli* Trx reductase (Sigma), 0.25 mM NADPH (Sigma) and purified protein (5.5 nM). After preincubation at 25°C for 2 min, the reaction was started by the addition of H_2O_2 (Wako Chemicals), t-butyl hydroperoxide (t-BOOH; Sigma) or cumene hydroperoxide (CM-OOH; Sigma) to the mixture at the final concentration of 0.3 mM, and the absorbance (340 nm) of the reaction mixture was monitored spectrophotometrically. For

measurement of GSH-dependent peroxidase activity, Trx and Trx reductase in the above-mentioned mixture were replaced with GSH (1 mM) and GSH reductase (5 units/ml), respectively.

Chemical modification of the protein

Free SH groups of the protein were modified by 4-acetamido-4'-maleimidyl-stilbene-2,2'-disulphonate (AMS) [18,19]. Purified proteins (100 µg) were incubated in 100 µl of the reaction mixture containing 100 mM DTT or 2 mM H₂O₂ in TE buffer at 20 °C for 20 min, and proteins were then precipitated with trichloroacetic acid at a final concentration of 5%. The precipitates were collected by centrifugation, washed with acetone, and dissolved in 20 µl of a freshly prepared solution containing 1% SDS, 50 mM Tris/HCl (pH 7.5) and 15 mM AMS by agitating at 20 °C for 30 min, followed by incubation at 37 °C for 10 min. Proteins were then separated by SDS/PAGE (15% gel) in non-reducing conditions and revealed by silver staining with Silver Stain KANTO III (KANTO Chemical, Tokyo, Japan).

Construction of an *E. coli* *bcp::kan* mutant

A 4.5-kb DNA fragment including the *bcp* gene was amplified from the chromosomal DNA of *E. coli* by PCR (upper primer, 5'-CGCTAACCACCTGCGGCCACAGAGTATTGC-3'; lower primer, 5'-GGCATGCTGCGGCTGTCCACGGCATCCG-3'). The PCR product was digested with *Sph*I, and the resulting *Sph*I fragment (3.8 kb) was purified and cloned into the *Sph*I site of pUC19 to yield pKD605. The kanamycin-resistance DNA cartridge from pUC4K (Pharmacia) was then inserted into the *Eco*RV-*Stu*I region of pKD605, which was located within the *bcp* gene, resulting in pKD606. The 5.0-kb *Sph*I DNA fragment of pKD606 containing *bcp::kan* was introduced into the cells of *E. coli* JC7623 (*recBC sbcBC*) by the standard electroporation procedure for *E. coli*, and kanamycin-resistant transformants were obtained. Chromosomal DNA of a kanamycin-resistant transformant (KD2300) was analysed by PCR and Southern-blot hybridization to verify the replacement of *bcp* by *bcp::kan*. The *bcp::kan* mutation was introduced into *E. coli* BL21 DE3 by P1 transduction, resulting in KD2301.

Agar diffusion assay for peroxide sensitivity

E. coli cells were grown in Luria broth containing 50 µg/ml ampicillin at 37 °C for 15 h. The culture was spread on Luria agar plates containing 50 µg/ml ampicillin and 1 mM IPTG, and a sterile filter-paper disc (7 mm in diameter) treated with 5 µl of 7% t-BOOH, 20% CM-OOH or 20% H₂O₂ was placed at the centre of each plate. The plates were incubated at 37 °C for 24 h, and the diameters of the growth-inhibition zones were measured.

RESULTS

Isolation of the *S. lineare* 17-kDa protein

Since we were interested primarily in plant DNases, the purification of the 17-kDa protein described in the Experimental section was guided by following a DNA-nicking activity up to the phosphocellulose step (results not shown). However, the major protein band in the final SDS/PAGE separation failed to exhibit such activity upon extraction followed by renaturation. Instead, subsequent gene cloning based on N-terminal sequence determination of the protein revealed that it was a homologue of *E. coli* Bcp (see below).

CAAAACCTAC AAACCTCATC TCAATCTCAG TTTCACGGCC TTAATTTCTC	50
Q T L Q T S S Q S Q F H G L K F S	
CCACGCTTCA TCTTTCAAAT CCCCGTCAGC TCCTTTGCGG AAAAATTCAA	100
H A S S F K S P S A P L R K N S I	
TCCTTGCTAA GGTTCAGAAA GGGTCGACGC CACGCGCTTT TACACTTAAG	150
F A K V T K G S T P P P F T L K	
GATCAGGAAG GGAGGCTGT AAGCTTGTC AAGTTTAAAG GAAAGCCAGT	200
D Q E G R P V S L S K F K G K P V	
AGTTGTGTAT TTCTATCTCG CCGATGAAAC TCCAGGATGC ACCAAGCAGG	250
V V Y F Y P A D E T P G C T K Q A	
CATGTGCATT CAGAGATTCG TACGAAAAGT TCAAGAAAGC AGGAGCTGAG	300
C A F R D S Y E K F K K A G A E	
GTGTGTGAA TAAGCGGAGA CAGTTCAGAG TCACACAAGG CATTTGCCAA	350
V V G I S G D S S E S H K A F A K	
GAAATACAG CTACCATTTA CGTTATTGAG CGATGAGGCG AACAGGTGA	400
K Y K L P F T L L S D E G N K V R	
GGAAAGAATG GGGAGTCCCG TCAGATTGT TTGGAACATT GCCTGGGAGG	450
K E W G V P S D L F G T L P G R	
GAAACATATG TTCTTGACAA GAATGGTGT GTTCAGCTTG TGTACAATAA	500
E T Y V L D K N G V V Q L V Y N N	
TCAGTTTCAA CCTGAAAAGC ATATAGACGA GACTTTGAAA TTAATTTCAGA	550
Q F Q P E K H I D E T L K L L Q S	
GCCTTAAATG AAGATTTCAT GCTTAGGTTT GTTTGTTTCA TTGTATCTGC	600
L K .	
TGTTTCATT ATCCTTGT TTGGTAAAC TTGTACATC GTTGTATGAG	650
AAAATTGA	658

Figure 1 Nucleotide and deduced amino acid sequences of the cloned *S. lineare* gene

N-terminal amino acid sequence of the purified 17-kDa protein is underlined. The sequence was deposited in the GenBank/EMBL/DBJ nucleotide sequence databases with the accession number AB037598.

Molecular cloning of the gene encoding the *S. lineare* 17-kDa protein

The N-terminal sequence for the 17-kDa protein recovered from the SDS/PAGE gel was determined to be KVTKGSTPPPT-LKDQEGRP, and oligonucleotide probes corresponding to the sequence FTLKDQE enabled us to isolate λgt11SLQ1 from a λgt11-based cDNA library of *S. lineare* by plaque hybridization. The inserted DNA (658 bp) of λgt11SLQ1 contained one open reading frame encoding a protein of 186 amino acids (Figure 1). We could not find an initiation codon in the 5'-end region of the open reading frame, but the N-terminal sequence of the 17-kDa protein mentioned above was found to start at the 37th residue from the 5' end of the insert DNA, indicating that the protein was produced by processing of its preprotein. The mature 17-kDa protein consisted of 150 amino acid residues, and its calculated molecular mass and isoelectric point were 16699 Da and 9.7, respectively.

Amino acid sequence similarity of the 17-kDa protein to the Bcp cluster

We searched the protein and DNA databases for homologues of the 17-kDa protein using the BLAST programs, and found that it shared high sequence similarities with the proteins in the Bcp

<i>S. cerevisiae</i>	MGEALRRSTR	IAISKRMLEE	EESKLAPIST	PEVPKKKIKT	GPKHNANQAV	VQEANRSSDV	NELEIGOPIE	DLSLL	75
<i>E. coli</i>	M-----	-----	-----	-----	-----	-----	NPLKNGDIAP	KFSLP	16
<i>S. typhimurium</i>	M-----	-----	-----	-----	-----	-----	NPLKNGDIAP	KFSLP	16
<i>K. pneumoniae</i>	M-----	-----	-----	-----	-----	-----	TPLKNGDIAP	KFSLP	16
<i>P. multocida</i>	M-----	-----	-----	-----	-----	-----	KTLSIGSSAP	QFSLP	16
<i>C. tepidum</i>	M-----	-----	-----	-----	-----	-----	ALLQNGQKAP	EFTAK	16
<i>C. difficile</i>	M-----	-----	-----	-----	-----	-----	--LSIGTKAP	EFTLE	14
<i>P. gingivalis</i>	M-----	-----	-----	-----	-----	-----	--IQIGDRIP	EILGI	14
<i>S. lineare</i>	K-----	-----	-----	-----	-----	-----	--VTIGSTIP	PFTLK	14
<i>S. cerevisiae</i>	NEDNDSISLK	KITENNRVVV	FFVYPRASF	* GCTRCACBFR	* DNYQELKKYA	-AVFGLSND	VTSQKNFQSK	QNLFP	149
<i>E. coli</i>	DQDGEQVNL	DF--QGQRVL	VYFYPKAMFP	GCTVQACBFR	DNMDELKKAG	VDVLGISTOK	PEKLSRFAEK	EILNF	89
<i>S. typhimurium</i>	DQDGEQVNL	DF--QGQRVL	VYFYPKAMFP	GCTVQACBFR	DNMDELKKAG	VDVLGISTOK	PEKLSRFAEK	EILNF	89
<i>K. pneumoniae</i>	DQDGEQVNL	DF--QGQRVL	VYFYPKAMFP	GCTVQACBFR	DNMDELKKAG	VDVLGISTOK	PEKLSRFAEK	EILNF	89
<i>P. multocida</i>	NQCKEIVSLR	QF--AGKKVL	VYFYPKAMFP	GCTVQACBFR	DSKTELEKRG	VVILGISTOK	PEKLSRFAEK	KALNF	89
<i>C. tepidum</i>	DQDGEVSLR	DY--TGRKVV	LYFYPKDDFP	GCTKEACAFR	DMLPNFEKVD	AVVLGISTOK	QKAHRFAEK	YILPF	89
<i>C. difficile</i>	DKDGMKVSMS	DF--KGKKVV	VYFYPKAMFP	GCTVQACBFR	MAYDGFKKED	IQVIGISTOK	IKSHQFAEK	EILPF	87
<i>P. gingivalis</i>	DQDGMKVRKD	DY--RGRKIA	LYFYPKAMFP	GCTVQACBFR	DSHSTLRAG	YEIIGISTOK	AKSHRFAEK	YILPF	87
<i>S. lineare</i>	DQEGRPVSL	KF--KGKPVV	VYFYPADEFP	GCTKQACAFR	DSYEKFKKAG	AEVVGISTOK	SESHKFAEK	YILPF	87
<i>S. cerevisiae</i>	ELLSPKREF	IGLLGAKTLP	LSGSIRSHFI	FVDGKLKFR	VKISPEV--S	VNDKKEVLE	VAEKFKEE-		215
<i>E. coli</i>	TLLSDDEHQV	CEQFGVWGEK	SFMGKTYDGI	HRISFLIDAD	GKIEHVF--D	DFKTSNHHDV	VLNWLKEHA		156
<i>S. typhimurium</i>	TLLSDDEHQV	CEQFGVWGEK	SFMGKTYDGI	HRISFLIDAD	GKIEHVF--N	DFKTSNHHDV	VLNWLKENA		156
<i>K. pneumoniae</i>	TLLSDDEHQV	CEQFGVWGEK	SFMGKTYDGI	HRISFLIDAD	GKIEHVF--D	DFKTSNHHDV	VLNWLKENA		156
<i>P. multocida</i>	ELLADDEHQV	AEQFGVWGEK	KFLGKTYDGI	HRISFLIDEQ	GMVEQVF--R	QFKTSNHHQV	VLDYLTQK-		155
<i>C. tepidum</i>	TLLVDDEKFI	VEAYGVWGLK	KFMGRYMG	NRVYLLIDEQ	GKIEKVF--S	KVKPETHAE	VLDWLQKKT		156
<i>C. difficile</i>	TLLSDPDIVA	IKAFDVWKEK	KMYGKTAGLV	VRATYIIDEN	GIIEKVF--E	KAKPDTHAQE	ILEYLEKQE		154
<i>P. gingivalis</i>	TLLVDDEKFI	NELFGVWVEK	SMYGRYMG	ERTTFLTDKE	GVVTRIIRGK	EVKTKDEADQ	ILNG----		151
<i>S. lineare</i>	TLLSDDEGNKV	RKEWGVPSD-	-LFG-TLPG-	-RETYVLDKN	GVVQLVYNNQ	-FQPEKHIDE	TLKLLQSLK		150

Figure 2 Comparison of the amino acid sequence of the 17-kDa protein with those of proteins in the Bcp cluster

Amino acid sequences were aligned with the use of GeneWorks software (IntelliGenetics). The conserved cysteine residues are indicated by asterisks. The accession numbers or websites for the sequences of Bcp-encoding genes are as follows: *Saccharomyces cerevisiae*, P40553; *E. coli*, M63654; *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pasteurella multocida*, *Chlorobium tepidum*, *Clostridium difficile* and *Porphyromonas gingivalis*, http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html.

cluster of the Prx family (Figure 2). The mature 17-kDa protein contained two cysteine residues, at positions 44 (Cys-44) and 49 (Cys-49). Both of these cysteine residues are well conserved in the proteins of the Bcp cluster, whereas only one cysteine residue (Cys-44) is conserved in this region of proteins from the other clusters of the Prx family [10,20,21]. Since these similarities suggested the possibility that the 17-kDa protein had the thiol-dependent peroxidase activity, we went on to examine the protein for its antioxidant properties.

Trx-dependent peroxidase activity of the recombinant 17-kDa protein

To investigate the enzymological properties of the 17-kDa protein, we constructed an *E. coli* overexpression system (BL21 DE3/pKD601) in which the gene encoding the protein was placed under the control of the T7 promoter (see the Experimental section). Crude extracts were prepared from IPTG-treated cells of this strain, and the recombinant 17-kDa protein was purified from the cell extracts by anion-exchange chromatography (Figure 3).

Trx-dependent peroxidase activity of the purified 17-kDa protein was clearly demonstrated: with the organic peroxide t-BOOH as a substrate, NADPH oxidation was dependent on the concentration of the purified protein (Figure 4a) and required the presence of Trx/Trx reductase (Figure 4b). H_2O_2 and the organic peroxide CM-OOH also served as substrates (Figure 5). We also investigated whether the 17-kDa protein had GSH-dependent peroxidase activity, with negative results.

Role of two conserved cysteine residues in the 17-kDa protein

To clarify the significance of the two conserved cysteine residues of the 17-kDa protein, we constructed two mutant proteins,

Figure 3 Purification of recombinant 17-kDa protein

Lane 1, the crude cell extracts of *E. coli* BL21 DE3 harbouring pKD601; lane 2, ammonium sulphate fraction; lane 3, anion-exchange chromatography fraction.

17K(C44S) and 17K(C49S), in which these residues were individually replaced by serine. These mutant proteins were overproduced in *E. coli* BL21 DE3 strains harbouring pKD603 and pKD604 (see the Experimental section), respectively, and purified to homogeneity as verified by SDS/PAGE. Neither 17K(C44S) nor 17K(C49S) showed Trx-dependent peroxidase activity in the assays using t-BOOH, CM-OOH or H_2O_2 as a peroxide (Figure 5). These results indicated that both of the cysteine residues were required for the peroxidase activity.

In some proteins of the Prx family, such as AhpC of *E. coli* and thiol peroxidase (TPx) of yeast, in which two cysteine residues are essential for the Trx-dependent peroxidase activity [21], one of these two residues is located at the site corresponding to Cys-44

Figure 4 Trx-dependent peroxidase activity of recombinant 17-kDa protein

(a) Effect of concentration of 17-kDa protein. 0 nM, ●; 1.25 nM, ○; 2.5 nM, △; 5 nM, ▲; 10 nM, ■. Reaction was started by addition of t-BOOH. (b) Requirements for NADPH oxidation. 17-kDa Protein, ○; Trx, ●; Trx reductase, △; or t-BOOH, ▲, was omitted from the complete reaction mixture with 5.5 nM 17-kDa protein (■).

of the 17-kDa protein, while the other is in the C-terminal region. These proteins are known to form a homodimer with an intermolecular disulphide bond under non-reducing conditions [8,19]. We investigated whether dimerization of the 17-kDa protein took place by subjecting it to SDS/PAGE under reducing and non-reducing conditions. The results revealed that it did not form a homodimer under non-reducing conditions, even after treatment with H_2O_2 (Figure 6a). This finding was confirmed by MALDI-TOF MS (Figure 6b).

Chemical modification of the 17-kDa protein with AMS

Chemical modification was achieved by alkylating the thiol groups of the reduced 17-kDa protein with AMS. Addition of the high-molecular-mass AMS moiety (500 Da) to the reduced but not to the oxidized protein allowed separation of these proteins by gel electrophoresis. As expected, we observed differently migrating forms of the 17-kDa protein depending on the treatment with H_2O_2 (Figure 7). The band *red 2*, which was observed in the lane for the reduced wild-type protein, represented the 17-kDa protein with two AMS-alkylated thiols. The band *red 1*, which was seen in the lanes for the reduced 17K(C44S) and 17K(C49S) mutants, represented the 17-kDa protein with one

Figure 5 Trx-dependent peroxidase activity of 17-kDa protein on various peroxides and its absence in the mutant proteins

The Trx-dependent peroxidase reaction was performed with 5.5 nM 17-kDa proteins and started by addition of t-BOOH (a), CM-OOH (b) or H_2O_2 (c). ●, Wild-type protein; ○, 17K(C44S); △, 17K(C49S).

AMS-alkylated thiol. The band *ox*, which was in the lanes for the H_2O_2 -oxidized 17-kDa proteins, represented the 17-kDa protein with no AMS-alkylated thiol. That oxidation of the 17-kDa protein abolished the reactive thiol groups strongly suggests that the two thiol groups of the 17-kDa protein formed a disulphide bond between them.

S. lineare 17-kDa protein complements the hypersensitivity of the *E. coli bcp* mutant to organic peroxides

Jeong et al. [13] found that an *E. coli bcp* mutant shows hypersensitivity to peroxides. To investigate whether *S. lineare* 17-kDa protein has the ability to suppress the defect of a *bcp* mutant, we determined the effect of its production on the peroxide sensitivity of an *E. coli bcp* mutant constructed as described in the Experimental section. A filter-paper disc treated with t-BOOH, CM-OOH or H_2O_2 was placed on a plate seeded with *E. coli bcp*

Figure 6 Absence of dimer formation of 17-kDa proteins under non-reducing conditions

(a) The wild-type protein (lanes 1–3), 17K(C44S) (lanes 4 and 5) and 17K(C49S) (lanes 6 and 7) were purified as described in the Experimental section. The purified protein samples (20 μ g of protein in 10 μ l) were mixed with the same volume of a reducing sample buffer [125 mM Tris/HCl (pH 6.8)/4% (w/v) SDS/20% (v/v) glycerol/10% (v/v) 2-mercaptoethanol; lanes 1, 4 and 6] or a non-reducing sample buffer (reducing sample buffer without 2-mercaptoethanol; lanes 2, 5 and 7), followed by 5-min incubation at 20 °C. After the heat treatment at 95 °C for 3 min, the samples were subjected to SDS/PAGE (15% gel). The wild-type protein in non-reducing buffer was also treated with H_2O_2 (final concentration, 3 mM) at 20 °C for 2 h. After removal of H_2O_2 with Microcon 10 (Amicon), the sample was heat-treated and subjected to SDS/PAGE (lane 3). (b) The wild-type protein in TE buffer was analysed by MALDI-TOF MS.

cells expressing the wild-type protein, 17K(C44S) or 17K(C49S). After 24 h of incubation at 37 °C, growth inhibition was evaluated by the size of the clear zone surrounding the disc (Figure 8). *E. coli bcp* cells (BL21 DE3 *bcp::kan*/pET11a) showed hypersensitivity to both of the organic peroxides (t-BOOH and CM-OOH), whereas *E. coli bcp* cells producing the wild-type 17-kDa protein showed the same sensitivity to both of the organic peroxides as the control *bcp*⁺ cells (BL21 DE3/pET11a). In contrast, *E. coli bcp* cells producing 17K(C44S) or 17K(C49S) showed the same sensitivities to the organic peroxides as those of the control *bcp* cells, except that the *bcp* cells producing 17K(C49S) showed partial resistance to t-BOOH. These results indicated that the 17-kDa protein could complement the hypersensitivity of the *bcp* mutant to the organic peroxides and that Cys-44 and Cys-49 were essential for this function. On the other hand, we observed no increase in sensitivity to H_2O_2 in the *bcp*

Figure 7 Chemical analysis of redox status of the 17-kDa protein

Purified 17-kDa protein (lanes 1 and 5), 17K(C44S) (lanes 2 and 6) and 17K(C49S) (lanes 3 and 7) were incubated in the reaction mixture containing dithiothreitol (lanes 1–3) or H_2O_2 (lanes 5–7) in TE buffer, treated with AMS and subjected to SDS/PAGE. Lane 4 shows untreated purified wild type 17-kDa protein.

Figure 8 Sensitivity to various peroxides of *E. coli* cells overproducing 17-kDa protein

E. coli BL21 DE3 harbouring pET11a (vector plasmid) (columns 1 and 6) and KD2301 (BL21 DE3 *bcp::kan*) harbouring pET11a (columns 2 and 7), pKD601 (*S. lineare* 17-kDa protein; columns 3 and 8), pKD603 [17K(C44S); columns 4 and 9] or pKD604 [17K(C49S); columns 5 and 10] were grown in Luria broth containing ampicillin for 15 h. The cells were spread on Luria-agar plates containing ampicillin and IPTG, and a paper disc treated with t-BOOH or CM-OOH was placed at the centre of each plate and incubated at 37 °C for 24 h. The diameters (in mm) of the clear zones around the discs were measured. Data are shown as the means $\pm$ S.D. from triplicate experiments.

mutant under the experimental conditions used here (results not shown).

DISCUSSION

The Prx family consists of a number of proteins that can be structurally and enzymically subclassified into several subfamilies [10,20]. Enzymically, members of the Prx family can be divided into two subgroups, 1-Cys Prx and 2-Cys Prx proteins [20–24]. One cysteine corresponding to Cys-44 of *S. lineare* 17-kDa protein is conserved in both 1-Cys and 2-Cys subgroups. Peroxidase activity of the 1-Cys Prx protein has been demonstrated by the glutamine synthetase-protection assay, in which a non-physiological reducing agent, dithiothreitol, is used as an electron

Scheme 1 Proposed peroxidase mechanisms for PrxQ, 1-Cys Prx and 2-Cys Prx

The mechanisms of 1-Cys Prx and 2-Cys Prx are according to Kang et al. [20]. Note that 1-Cys Prx does not use Trx as an electron donor but instead has an as-yet-unidentified electron donor (XH_2). Closed circles indicate the N-terminus of each protein.

donor. However, the 1-Cys Prx protein does not show Trx-dependent peroxidase activity. Oxidation of the conserved cysteine residue of a 1-Cys Prx is responsible for the antioxidant function, and the oxidized cysteine residue is reduced by an unknown reducing agent(s) under physiological conditions [20,25] (Scheme 1). On the other hand, members of the 2-Cys Prx subgroup have two conserved cysteine residues. Typically, one (the first Cys) is located at the same position as the conserved cysteine of 1-Cys Prx and the other (the second Cys) is in the C-terminal region. The 2-Cys Prx protein shows peroxidase activity by both the glutamine synthetase-protection assay and the Trx-specific assay with the enzymic thiol-regenerating system (Trx/Trx reductase/NADPH) [21,26]. The first Cys is essential for peroxidase activity in both assay systems, whereas the second Cys is required only for the Trx-dependent peroxidase reaction. The first Cys of a molecule can bind to the second Cys of another through an intermolecular disulphide bond, resulting in the formation of a homodimer (Scheme 1).

In this study, we identified in *S. lineare* a 17-kDa protein homologous with *E. coli* Bcp. We demonstrated further that the 17-kDa protein possessed Trx-dependent peroxidase activity, a finding to justify the classification of the protein in a discrete cluster within the Prx family consisting of Trx-dependent per-

oxidases. Since the name Bcp gives no functional insight, we would like to designate the *S. lineare* 17-kDa protein PrxQ. Thus far, Bcp homologues have been reported only in bacteria and unicellular eukaryotes. Hence, PrxQ represents the first member in the Bcp cluster to be found in a higher organism. Judging by its cDNA sequence, PrxQ appears to be processed from a precursor protein. The deduced amino acid sequence upstream of the N-terminus of the mature PrxQ has some characteristics of transit peptides: high amounts of the hydroxy amino acids serine and threonine and the positively charged amino acids arginine and lysine, and a lack of tyrosine and negatively charged amino acids [27,28], indicating that PrxQ is likely to be in the plastids of the plant.

The two cysteine residues (Cys-44 and Cys-49) identified in PrxQ are well conserved among the proteins in the Bcp cluster. Based on the results with the mutant PrxQ proteins (C44S and C49S), it was concluded that these two cysteine residues were essential for the Trx-dependent peroxidase activity. Since a protein disulphide bond can be reduced by Trx [5,29,30], it is likely that the two closely situated cysteine residues of PrxQ form an intramolecular disulphide bond in presence of peroxides. This notion was supported by the observations that PrxQ did not form a homodimer under non-reducing conditions even after H_2O_2 treatment and that H_2O_2 treatment abolished the AMS-reactive thiol groups of PrxQ. We hypothesize that one Cys-SH (probably Cys-44) is oxidized directly by peroxides to form cysteinesulphenic acid (Cys-SOH) and that the Cys-SOH reacts with the second Cys-SH of the same molecule to form a disulphide bond. This disulphide bond can be reduced by electrons from Trx. This reaction scheme resembles those for the redox proteins such as Trx, glutaredoxin, Dsb and Trx reductase [31–36]. In a sense, PrxQ may be regarded as a class of 2-Cys Prx because the two conserved cysteine residues are likely to form a disulphide intermediate that is subject to reduction by Trx. However, it differs from the classical 2-Cys Prx proteins such as AhpC of *E. coli* and TPx of yeast in that the two conserved cysteine residues are not far apart and that intramolecular rather than intermolecular disulphide bonds are formed upon oxidation.

In contrast with the second Cys of PrxQ being essential for Trx-dependent peroxidase activity (this study), a mutant Bcp that possessed a serine residue in place of the second Cys had the activity, although it was lower than that of the wild-type Bcp [13]. PrxQ has only two cysteine residues located in the N-terminal region, whereas Bcp has three cysteine residues: two located at the sites corresponding to those of PrxQ and another in the C-terminal region. Presence of the third cysteine residue in Bcp may explain the difference in the significance of the second Cys between PrxQ and Bcp.

Examination of *E. coli* cells expressing PrxQ for sensitivity to peroxides suggests that *S. lineare* PrxQ may function *in vivo* as an antioxidant. Wild-type PrxQ suppressed the hypersensitivity of the *E. coli* bcp mutant to the organic peroxides t-BOOH and CM-OOH, whereas the C44S and C49S mutant proteins did not. Interestingly, although the C49S mutant of PrxQ was inactive as a Trx-dependent peroxidase, the *E. coli* bcp mutant expressing it showed partial resistance to t-BOOH. This may be explained by assuming that this mutant PrxQ acts as a 1-Cys Prx *in vivo* with some unknown substance serving as an electron donor. In this context, it is known that in the classical 2-Cys Prx proteins the cysteine residue in the C-terminal region, which is essential for Trx-dependent peroxidase activity, is dispensable in Trx-independent peroxidase activity [21,22,37].

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