Cloning, Overexpression, and Characterization of Peroxiredoxin and NADH Peroxiredoxin Reductase from *Thermus aquaticus*†

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The genes for peroxiredoxin (Prx) and NADH peroxiredoxin oxidoreductase (PrxR) have been cloned from the thermophilic bacterium *Thermus aquaticus*. prx is located upstream from prxR, the two genes being separated by 13 bases. The amino acid sequences show that Prx is related to two-cysteine peroxiredoxins from a range of organisms and that PrxR resembles NADH-dependent flavoenzymes that catalyze the reduction of peroxiredoxins in mesophilic bacteria. The sequence of PrxR also resembles those of thioredoxin reductases (TrxR) from thermophiles but with an N-terminal extension of about 200 residues. PrxR has motifs for two redox-active disulfides, one in the FAD-binding site, as occurs in TrxR, and the other in the N-terminal extension. The molecular masses of the monomers of Prx and PrxR are 21.0 and 54.9 kDa, respectively; both enzymes exist as multimers. The recombinant flavoenzyme requires 3 mol equivalents of dithionite for full reduction, as is consistent with 1 FAD and 2 disulfides per monomer. PrxR and Prx together catalyze the anaerobic reduction of hydrogen peroxide. The activity of Prx is much less than has been observed with homologous proteins. Prx appears to be inactivated by cumene hydroperoxide. PrxR itself has low peroxidase activity.

Peroxiredoxins (Prx)† are a group of small proteins that catalyze the reduction of hydrogen peroxide to water and of alkyl hydroperoxides to the corresponding alcohols (1–3). They have been identified in many organisms from bacteria to mammals and implicated in a wide variety of cellular processes including proliferation (4), differentiation (5), and the immune response (6) as well as in the detoxification of peroxides. They have been given various names, including thiol-specific antioxidant (7), heme-binding protein 23 (HBP 23) (8), and alkyl hydroperoxide reductase (9). They occur as homo-dimers and higher multimers of monomers of molecular mass 19 to 29 kDa. The active site at which peroxide is reduced includes a cysteine residue toward the N terminus of the protein whose side chain is thought to be oxidized to a sulfenic acid during the reaction (10, 11). It appears that in many peroxiredoxins the sulfenic acid reacts with the thiol of a second conserved cysteine residue toward the C terminus of the other monomer to form a cystine disulfide, which is subsequently reduced back to the dithiol. The second cysteine is not present in some peroxiredoxins from higher organisms, and in these cases, the sulfenic acid is reduced directly to the thiol (11). The external reductant for peroxiredoxins varies with the source of the enzyme, but it is invariably a thiol-containing molecule such as cysteine or trypanothione (3) or, more commonly, the protein thioredoxin (Trx) in combination with NADPH thioredoxin reductase (TrxR) (12). In bacteria the reducing equivalents are provided by an NAD(P)H-dependent flavoenzyme in which a reduced cysteine disulfide is the direct electron donor to peroxiredoxin (10, 13, 14). The first enzyme to be recognized in this group was isolated from *Salmonella typhimurium* and termed AhpF (9). Similar enzymes from other sources were sometimes first isolated as NADH dehydrogenases or NADH oxidases, and their roles in peroxiredoxin reduction only became apparent later. Poole et al. (15) recently proposed the name NADH:peroxiredoxin oxidoreductase or peroxiredoxin reductase (PrxR) for enzymes in this group, a name that is adopted in this paper. Electron transfer in the bacterial systems occurs as follows: NADH → PrxR → Prx → H₂O₂.

A flavoenzyme in the thermophilic bacterium *Thermus aquaticus* was first isolated as an NADH oxidase (16). More recently, several properties of the enzyme, including its chemical composition and its ability to catalyze cumene hydroperoxide reduction in the presence of Prx from *S. typhimurium*, suggested that it is related to NADH peroxiredoxin reductases from other organisms (17, 18). These observations implied that a corresponding peroxiredoxin is synthesized in *T. aquaticus*. The N-terminal amino acid sequence of a small protein that was found to copurify with the flavoenzyme was shown to resemble that of peroxiredoxins from other bacteria (18). However, it proved difficult to obtain the putative peroxiredoxin free from the flavoenzyme. Since large amounts of both proteins are required for studies on their structures and catalytic mechanisms, we have cloned their genes, overexpressed them separately in *Escherichia coli*, purified the recombinant proteins, and characterized them biochemically. This paper describes the first peroxiredoxin system from a thermophile and shows that the two enzymes comprise a system similar to the peroxiredoxin-dependent systems of other bacteria. A preliminary report has been published on the cloning of the two genes (19).

MATERIALS AND METHODS

*Growth of Bacteria—* *T. aquaticus* YT-1 (NCIMB 11243) was cultured as described previously (18). *E. coli* strains TG1, DH5α, and BL21(DE3)
were maintained and propagated in Luria-Bertani medium (20) in cultures of up to 4 liters. The growth medium was supplemented with ampicillin (100 μg/ml) or kanamycin (30 μg/ml) according to the vector used and with IPTG (1 mM added in log phase, A₅₅₀ = 0.5) when induction of the synthesis of PrxR was required.

Protein Purification—Native PrxR and Prx were purified from T. aquaticus with the following modifications to the published method for the flavoprotein (18). DNase (30 μg ml⁻¹) was included in the buffer used to suspend the cell paste. Elution of the flavoprotein from AMP-Sepharose with a pH gradient gave yellow fractions that also contained Prx as the only contaminant, as judged by SDS-PAGE analysis. The fractions were combined, concentrated by ultra-filtration (Amicon Corp.; PM10 membrane), dialyzed versus 0.1 M potassium phosphate buffer, pH 6.0, containing 0.5 M NaCl, and applied to a column of Sephacryl 200 (82 cm x 1.6 cm diameter) equilibrated with the same buffer. The first fractions from this gel filtration column contained both proteins, whereas a second band of protein contained only the flavoprotein. Material from the second band was used as the source of native PrxR; the mixture of PrxR and Prx was used to compare the catalytic activities of native and recombinant Prx.

Recombinant T. aquaticus PrxR was purified as follows from E. coli BL21(DE3) that had been transformed with pDK6/PrxR. All buffers contained 0.3 M EDTA. A crude extract of E. coli was made by suspending 9.3 g of cell paste in 30 ml of 20 mM Tris-HCl, pH 7.4 (buffer A). Bovine serum albumin (5 mg/ml) was added, sonication (Branson sonicator tube, operated at 80% full power), and centrifuging (23,500 x g for 20 min). The extract was mixed with DEAE cellulose (300 ml of a slurry of Whatman DE32 that had been allowed to settle from buffer A), and the mixture stirred for 1 h. The DEAE-cellulose was separated by filtration on a filter funnel fitted with a sintered disc and washed in the funnel with 1.5 liters of buffer A plus 0.15 M NaCl. The PrxR was then eluted with 0.45 M NaCl in buffer A, and the yellow solution was dialyzed for 12 h against 0.1 M potassium phosphate, pH 7.0 (buffer B), using three changes of buffer. The solution was applied to a column of AMP-Sepharose (12 x 1.5-cm diameter) equilibrated with buffer B. The column was washed with 120 ml buffer B, leaving the enzyme in a yellow band at the top of the column. A pH gradient was applied to the column by continuous dilution of 150-ml buffer B with 150 ml of 0.1 M Tris-HCl, pH 9.0 (buffer C). This eluted part of the enzyme. The remainder was eluted with more buffer C. Fractions (2 ml) were collected and analyzed by SDS-PAGE. Those that contained only PrxR were pooled, concentrated by ultrafiltration as described above, and stored at -20 °C.

Recombinant Prx was purified as follows from E. coli BL21(DE3) that had been transformed with pRSETB/Prx. All buffers contained 0.5 mM EDTA. Cell paste (20.5 g) was suspended in 400 ml of cell lysis solution (50 mM Tris-HCl, 40 mM EDTA, pH 8.0, and 1 mg/ml lysozyme). After incubating the mixture on ice for 30 min, the suspension was sonicated and centrifuged as described for PrxR. The supernatant was incubated at 70 °C in a water bath for 30 min, then cooled to 4 °C and centrifuged (20,000 x g, 20 min). The precipitate was discarded, and the supernatant was dialyzed (0.2-0.3 ml) against 0.1 M potassium phosphate, pH 7.0, containing 0.5 M NaCl, and 0.3 mM EDTA at a flow rate of 0.5 ml/min. The dialyzed solution was applied to a DEAE-sepharose column, and the elution was monitored, in 3 ml, 50 mM potassium phosphate buffer, pH 7.0, 0.3 mM EDTA, 0.45 μM methyl violenol to promote electron transfer, and flavoenzyme. A side arm on the cuvette contained 0.4 mM of 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA, 0.3 mM methyl violenol, and 3 μM 3-methyl-5-deaza lumiflavin. The cuvette was made anaerobic by repeated cycles of evacuation and filling with purified nitrogen. The methyl violenol in the side arm was then photo-reduced to act as a sink for residual oxygen and as a visible check that the cell remained anaerobic. A solution of sodium dithionite (0.1 mM sodium pyrophosphate-HCl buffer, pH 9.0) that had been standardized with FMN was added stepwise to the cuvette (21). An absorption spectrum was recorded after each addition when all changes in the visible spectrum were complete.

Analytical Methods—Protein was determined by the dye binding method using bovine serum albumin as the standard (22). The quantitative extraction of FAD from recombinant PrxR was achieved by incubating the enzyme in a sealed tube at 100 °C for 12 min (23). The protein precipitated during the heat treatment, and it was subsequently removed by centrifugation (20,500 x g, 10 min) before measuring the absorption spectrum of the supernatant.

Protein size determination was performed using a calibrated FPLC column of Superdex-200 HR (Amersham Pharmacia Biotech) to analyze the apparent molecular mass of the purified native and recombinant proteins under non-denaturing conditions. The column (30 cm x 1-cm diameter) was equilibrated with 50 mM potassium phosphate, pH 6.0, containing 0.15 M NaCl and 0.3 mM EDTA at a flow rate of 0.5 ml/min. The molecular mass of the elution was monitored at 280 nm. SDS-PAGE was performed (24) using a vertical slab gel (ATT AE-6450). The separated proteins were stained with Coomassie Blue.

DNA Manipulations—Cloning and transformation techniques were carried out as described (20). Plasmid DNA was isolated by the alkaline lysis method (20). T. aquaticus genomic DNA was isolated and purified from cultures that were grown for no more than 16 h (25).

The polymerase chain reaction (PCR) was used together with a Techne thermocycler to amplify genomic DNA. Reactions were carried out in a reaction volume of 100 μl that contained 0.1 μg of genomic DNA, either primers A and B or primers C and D as given below (1 μM each), and 2 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, the four dNTPs (0.25 mM each), and 5 units of Taq polymerase (Sigma). Glycerol (30 μl 50% (v/v)) was included in amplification reactions in which primers A and B were used. The DNA was denatured by incubating at 95 °C for 5 min before adding it to the reaction. The conditions used were as follows: 40 cycles of denaturation at 95 °C for 1.5 min, annealing at 40 °C for 1.5 min, and extension at 72 °C for 3 min. Primer A was AATGCGGAGTAYAHRGC. Primer B was ATGGCTCTC TCNCC. Primer C was GCGAAAAATCCTTGCCGATCATTG CGC. Primer D was ATGGGNNAAARGTNACCC. The regions that are underlined at the 5’ ends of the primers were included to increase the stability of the priming duplex. The reaction products were analyzed by agarose gel electrophoresis. They were isolated from the gel using the GeneClean kit (Bio 101) and subcloned into the pCR® 2.1 plasmid (Invitrogen) to generate the plasmids pCR2.1PRX990 and
Identification and Cloning of PrxR and Prx Genes—The DNA fragments amplified by PCR were used as probes in Southern blot analysis. The probes were labeled with digoxigenin-11-dUTP in a random-primer labeling reaction (Roche Molecular Biochemicals kit, 1175033). Purified genomic DNA was digested with a range of restriction enzymes, both singly and in pairs, to generate fragments that could be screened for both PrxR and Prx. Restriction mapping of PRX600 PRXR990 identified several restriction sites that were used in the mapping of the complete Prx and PrxR (Fig. 1). The restriction digests were separated by electrophoresis in 0.8% agarose gel and transferred to positively charged nylon membranes (26). DNA fragments that contained the genes encoding both PrxR and Prx were identified by direct hybridization of the blots with the digoxigenin-11-dUTP-labeled probes and subsequent detection in the specific antibody-mediated chemiluminescence reaction. Larger amounts of DNA were digested with the enzyme PstI, which produced single-sized fragments of DNA that hybridized to both probes and were therefore likely to contain the full-length genes. Size-selected fragments were isolated from agarose gel and ligated into plBluescript SK− (Stratagene) that had been digested with the same enzyme and treated with calf-intestinal alkaline phosphatase (New England Biolabs) to prevent self-ligation. The ligated products were transformed into E. coli TG1, and the plasmid DNA was isolated from colonies that contained recombinant plasmids. These plasmid preparations were screened by restriction mapping, Southern blot analysis, and DNA sequencing. A plasmid that contained the full-length PrxR and Prx genes in a 3.8-kb fragment was identified and termed plBluescript/PfPrxR.

Expression of Recombinant Genes—prxR was subcloned into pDK6 (27) to give the expression plasmid pDK6/PrxR. The prx gene was similarly subcloned into pRSETB (28), giving the expression plasmid pRSETB/Prx. The full-length genes were amplified (from start codon to stop codon) by PCR with the high fidelity polymerase Pfu (Stratagene) and using non-degenerate primers, which incorporated appropriate restriction sites to facilitate subcloning. Reactions were carried out in a reaction volume of 100 μl that contained 50 ng of plBluescript/PfPrxR plasmid DNA, either primers PrxRNterm and PrxRCTerm (for prxR amplification) or primers PrxNterm and PrxCterm (for prxR amplification) as given below (0.5 μM of each), 20 μM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM ammonium sulfate, 20 μM magnesium sulfate, 0.01% Triton® X-100, 0.01 mg/ml bovine serum albumin, the four dNTPs (0.25 mM each), 200 μM of dGTP, 5 units of cloned plasmid DNA polymerase. The conditions used for primers PrxRNterm and PrxRCTerm were as follows: 30 cycles of 94 °C for 0.75 min, annealing at 50 °C for 0.75 min, and extension at 72 °C for 4 min. Reaction conditions for amplification with primers PrxNterm and PrxCterm were 30 cycles of 94 °C for 0.75 min, annealing at 55 °C for 0.75 min, and extension at 72 °C for 2 min. All reactions were terminated with a 10-min cycle at 72 °C. Primer PrxRNterm incorporated a restriction site for restriction enzyme EcoRI (GGATC) upstream of ATGCCGACATT. Primer PrxCterm incorporating a PstI restriction site was CTGCAAGTTATGCCGACATTCAAAATATC. Primer PrxNterm incorporating an NdeI restriction site was GGGAATTCCATCATATGCTTGTGGGGAAAA. Primer PstIcterm incorporating a PstI restriction site was CTGCAAGTTATGCCGACATTCAAAATATC. The restriction sites (underlined, with an underlining the points of cleavage) allowed the PCR products to be cloned into the required expression vector. DNA isolation and manipulation procedures involved the use of the GeneClean kit for isolation of amplified products from agarose gels, restriction digestion of the purified PCR products and expression vectors, and other standard procedures for subsequent cloning of DNA fragments (20). Following transformation of E. coli BL21(DE3) with the appropriate expression construct (pDK6/PrxR or pRSETB/Prx), cultures of the bacterium were grown at 37 °C in the presence or absence of IPTG as required. Crude lysates of E. coli were made to determine the levels of expression under a variety of growth conditions. The lysates were made by resuspending the cell pellets in cell lysis solution and incubating as outlined above. After centrifugation (18,500 × g, 20 min) the supernatant was incubated at 70 °C for 30 min. After a further centrifugation (18,500 × g, 20 min) the supernatant was analyzed by SDS-PAGE using 12.5% gels and Coomassie Blue staining.

Computer-based Methods—DNA sequence analysis was carried out using the Macmolpy package (Sofigene, Berlin). The amino acid sequences were aligned using ClustalW (29). The secondary structure of the polypeptide sequences was determined using Predict Protein (30–32).
sequenced and found to contain the complete genes for PrxR and Prx. This plasmid was designated pBluescript/PrxPrxR.

The 3833-base pair insert from pBluescript/PrxPrxR was sequenced on both strands. ORF Finder (NCBI) identified one partial and three complete open reading frames that are homologous to entries in the GenBank®, Swiss-Prot, or PIR data bases (BLAST search algorithm (33)) (Fig. 1). The amino acid sequence of the Prx is derived from bases 1–561 (Fig. 2). A putative ribosome-binding site similar to those found in *E. coli* is positioned 10 bases upstream from the initiation codon. The gene is initiated by an ATG start codon and is terminated by a single TAA stop codon. It encodes a polypeptide with 187 amino acids and a molecular mass of 20,982 Da. The gene that encodes PrxR is separated from *prx* by 13 bases. The amino acid sequence of PrxR is derived from bases 578–2104 (Fig. 2). This gene is also preceded by a putative ribosome-binding site (bases 562–569) 8 bases upstream from the initiation codon, and it is terminated by a single TAA stop codon. It encodes 509 amino acids, and the polypeptide has the molecular mass 54,863 Da. The two genes are translated in different reading frames, indicating that although they might be transcribed as a single polycistronic mRNA molecule, they are translated as individual polypeptides.

A third open reading frame was identified from bases −955 to −491, encoding a polypeptide with 154 amino acids (Fig. 1). The gene is initiated by a single ATG start codon and is terminated by a single TGA stop codon. The sequence of the proposed gene product is most similar to conserved hypothetical proteins from *Bacillus subtilis* (A69881) and *Thermotoga maritima* (E72338) (72 and 58% identity, respectively). A partial open reading frame was identified from bases 2587–2679 encoding a polypeptide with 31 amino acids (Fig. 1). A single ATG codon initiates the gene that is present in the DNA sequence in the same reading frame as the *prxR*. A search of the protein data bases showed sequence identity of the partial gene product with the N-terminal sequences of five bacterial histidyl tRNA synthetases. The sequence is most similar to the proteins from *B. subtilis* and *Staphylococcus aureus* (68 and 57% identity, respectively) and to which it is related by many conservative amino acid substitutions (80 and 95%, respectively).

The G/C contents of *T. aquaticus* *prxR* and *prx* are 53.1 and 51.1%, respectively. There is a slight bias in favor of G/C in the third codon position (62.9 and 60.1% for *prxR* and *prx*, respectively). The G/C content in the first and second codon positions is 59.4 and 38.6%, respectively, for *prxR* and 56.9 and 36.1%, respectively, for *prx*. The G/C content of the two genes is less than the overall G/C content of other coding sequences in *T. aquaticus* DNA. The overall content is 66.6% as determined for 65 coding sequences that contain 21,160 codons, with a bias for G/C in the first, second, and third codon positions of 67.8, 43.0, and 88.9% respectively (GenBank®, codon usage data base).

### Amino Acid Sequence Comparisons

A search of the sequence data bases using BLAST (33) showed that the amino acid sequences of PrxR and Prx from *T. aquaticus* are similar to those of corresponding proteins from other organisms (Table I). The sequence of *T. aquaticus* Prx is most similar to the sequences of Prx (AhpC) from *Amphibacillus xylanus*, *B. subtilis*, and *Streptococcus mutans* (77, 75, and 73% identity, respectively). The *T. aquaticus* protein contains three cysteine residues. Two of them (positions 47 and 166) are conserved in the proteins from other organisms, where they are known to be redox-active and to function in the peroxidase activity. The third cysteine (position 37 in *T. aquaticus* Prx) is present in the *B. subtilis* and *S. mutans* proteins but not in the *A. xylanus* Prx (AhpC) or in the well characterized *S. typhimurium* protein, which shares 67% identity with *T. aquaticus* Prx. The sequence of the *T. aquaticus* protein is also similar to the sequences of peroxiredoxins in eukaryotes. For example the identity with the 2-eyes Prx from *Arabidopsis thaliana* is 43%.

The amino acid sequence of the flavoprotein from *T. aquaticus* is similar to the sequences of flavoproteins from a variety of bacteria that have been variously identified as NADH dehydrogenases, NADH oxidases, and/or components of alkyl hydroperoxidases termed AhpF. Table I shows that the greatest similarity is with the enzymes in *B. subtilis*, *Bacillus alcalophilus*, and *A. xylanus*. As noted earlier, the name NADH:peroxiredoxin oxidoreductase (PrxR) has recently been proposed for enzymes in this group (15). A lower level of sequence identity is observed with bacterial thioredoxin reductases, most notably with the proteins identified from the thermophilic bacteria *Pyrococcus abyssi* and *Thermotoga maritima* (Table I). Sequence alignment shows that the PrxRs contain an N-terminal extension of approximately 200 amino acids that is not present in the NADPH thioredoxin reductases. The motifs for two pairs of redox-active cysteines, CXXC, occur in *T. aquaticus* PrxR (residues 128–131 and 337–340). The NADPH thioredoxin reductase proteins contain only one of these motifs, and it corresponds with the more C-terminal of the two motifs in PrxR.

One class of flavoproteins that contain FAD and a single pair of redox-active cysteines involved in the transfer of reducing equivalents from the FAD cofactor to the substrate (class II pyridine nucleotide disulfide oxidoreductases, PNDRII) is characterized by the active site fingerprint sequence: **CX,CDG/AI,XX**, **(F/Y)XX(L/V/M),XX(L/V/M),G** if defined by PROSITE, PD004965 (34). The class includes prokaryotic and eukaryotic thioredoxin reductases (35, 36) and bacterial PrxRs (37). The sequence is present in *T. aquaticus* PrxR between residues 337 and 357 (Fig. 3). The *T. aquaticus* PrxR also contains two motifs that correspond to the consensus sequence for the binding site of ADP (38, 39) and that are possible
binding sites for the ADP moieties of FAD and NAD(P)H. They contain three conserved glycine residues flanked by small hydrophobic residues, with an acidic residue occurring at the C terminus of the sequence (V/I/L/A)3G(A/G/S)GG(A/G/I/L/S)2G(A/S)(A/G/I/S/V)X(G/I/L/M/V)(D/E).

Furthermore, the predicted secondary structures surrounding these two motifs are in the form of bab folds, as is diagnostic of nucleotide binding sites (Fig. 3). The amino acid sequence of T. aquaticus PrxR (residues 469–479) is in complete agreement with the proposed consensus sequence motif for the binding site of the FAD flavin moiety (38).

A comparison of the amino acid compositions of proteins from thermophiles with those from their mesophilic homologs has shown that thermostable proteins tend to have a greater content of charged and hydrophobic amino acids and a smaller content of uncharged polar amino acids (40). Grouping of the amino acids of T. aquaticus Prx and PrxR into these three classes shows that they do not differ significantly from the corresponding groups for the nine most homologous proteins from mesophiles that are listed in Table II.

Overexpression of Recombinant PrxR and Prx—A major aim of the present investigation was the expression in E. coli of the recombinant genes for the T. aquaticus proteins and purification of the two proteins in amounts sufficient for biochemical characterization. Neither gene is expressed from the plasmid pBluescript/PrxPrxR in which the 3.8-kb insert is not correctly oriented for transcriptional control to be exerted by the lac promoter. It was shown that this plasmid does not survive in E. coli during culture of the organism at 37 °C in the presence or absence of IPTG (0.1–1.0 mM). The genes for the two proteins were therefore separately amplified using the high fidelity polymerase Pfu, with simultaneous incorporation of appropri-
FIG. 3. Consensus sequences for nucleotide binding and active sites and their secondary structures in T. aquaticus PrxR and homologous proteins. A, proposed pyridine nucleotide disulfide reductase class II active site. B and C, the proposed binding sites for the ADP moieties of FAD and NAD(P)H respectively. D, the proposed binding site for the FAD flavin moiety. The amino acid positions are those in binding site for the FAD flavin moiety. The amino acid positions are those in B. subtilis (accession numbers P42974) and B. alcalophilus (accession number A49911) were aligned with the T. aquaticus Peroxiredoxin and NADH Peroxiredoxin Reductase.

The gene prxR was cloned into the NdeI (5′-end) and PstI (3′-end) sites of pRSETB/Prx, an ampicillin-resistant vector with transcription regulated by the T7 promoter. The greatest expression of the soluble form of this protein was also observed in the BL21(DE3) strain of E. coli, but in this case, in the absence of IPTG. It was observed that in the presence of IPTG, much of the recombinant protein was insoluble. Overexpression of Prx from pRSETB/Prx was not observed in E. coli TG1. Expression of Prx was also investigated in the expression vectors pKK223–3 (41) and pDK6 (27). Prx was inserted into the XmaI (5′-end) and PstI (3′-end) sites of both vectors, and protein expression was examined in strains BL21(DE3) and TG1 of E. coli. Analysis of cell lysates by SDS-PAGE showed that overexpression of Prx did not occur.

Purification of PrxR and Prx—Recombinant T. aquaticus Prx was purified from E. coli grown in a 4-liter culture. The cell extract was heat-treated to denature and precipitate most of the E. coli protein. The recombinant protein from the thermophile remained in solution, where it composed about 95% of the protein. The absorbance maximum of the sample after heat treatment was at lower wavelength (265 nm) than is usual for protein. The main residual contamination was DNA, and this was not completely separated by chromatography on DEAE-cellulose or by a subsequent precipitation of the protein with ammonium sulfate. The remaining DNA was therefore removed from the protein by gel filtration. After this step the protein was judged by SDS-PAGE to be at least 99% pure (Fig. 4). Approximately 144 mg was obtained from 20.5 g of cell paste. A specific assay for Prx was not used during the three steps of the fractionation, the purification being monitored by measurement of the UV absorption spectrum and SDS-PAGE analysis. Therefore, the overall recovery of this recombinant enzyme is not known; it is estimated to be about 50%.

Recombinant PrxR was purified from cell paste from a 2-liter culture of E. coli using two chromatographic steps similar to those used for the native enzyme. It was not possible to initiate the purification of this enzyme with heat denaturation because the treatment led to incomplete binding of the enzyme to the AMP-Sepharose used in the following step. The flavoenzyme was therefore separated from the extract using DEAE-cellulose as used previously with the native enzyme (18). Approximately
for Prx and PrxR, respectively (Fig. 4). These values are in close agreement with the values calculated from the derived amino acid composition of 21.0 and 54.9 kDa for the Prx and PrxR, respectively. Analysis of the pure recombinant proteins by FPLC in a column of Superdex 200 produced a single peak in each case with an apparent molecular mass of 235 kDa for Prx and 130 kDa for PrxR (Fig. 4), implying that both proteins occur as multimers. Solutions of Prx were colorless, the absorption spectrum showing a single band with a maximum at 280 nm. A value for the molar absorption coefficient at the maximum was calculated from the amino acid composition and the molecular mass of the protein using the program ProtParam ($\epsilon_{280} = 26,200 \text{ M}^{-1} \text{cm}^{-1}$). The yellow solution of recombinant PrxR has absorbance maxima at 270, 384, and 450 nm. The ratio $A_{270}/A_{384}$ is 6.1, similar to that reported previously for the native enzyme. The flavin in the supernatant after heat denaturation of the apoenzyme was shown to be FAD. This quantitative extraction of the flavin also confirmed an earlier report with the native enzyme (18) that the visible absorbance of the enzyme-bound FAD is greater than that of FAD in free solution ($\epsilon_{450} = 11.3$ (42) and 13.0 $\text{mM}^{-1} \text{cm}^{-1}$ for free and bound FAD, respectively). In further contrast to FAD, the absorbance at 384 nm for the enzyme-bound flavin is greater than the absorbance at 450 nm ($A_{384}/A_{450} = 0.92$). The FAD content of the recombinant protein was determined using the absorption coefficient at 450 nm, protein analysis using a modification of the Bradford assay, and the value 55,692 Da for the molecular mass of the subunit of PrxR calculated from the derived amino acid composition plus a molecule of FAD. The flavin content of the isolated recombinant PrxR (0.98 mol of FAD/subunit) is in close agreement with the flavin content of the native enzyme (18).

The anaerobic spectrophotometric titration of recombinant PrxR with sodium dithionite showed that the enzyme requires approximately 6 electrons to fully reduce the FAD to the hydroquinone and confirmed earlier observations on the native enzyme that the reduction in such static titrations occurs in several phases (Fig. 5). In the first phase, during the stepwise addition of approximately 0.8 mol of dithionite/mol of enzyme FAD, the flavin undergoes a rapid reduction after each addition of reductant. The flavin spectrum then slowly changes to give a spectrum that in the long wavelength region is characteristic of the blue neutral form of the semiquinone. At the end of this phase, approximately 11% of the flavin is in form of semiquinone, as judged by the absorbance at 580 nm ($\epsilon_{580} = 4.3 \text{ mM}^{-1} \text{cm}^{-1}$). In the second phase of the titration, during the further addition of almost 2 mol of dithionite/mol of FAD, the semiquinone increases to a maximum. During this phase the initial rapid reduction of the flavin after each addition of dithionite is followed by a slow further reduction of the flavin. At the end of this second phase 94% of the flavin was found to be in the form of the semiquinone. The third phase involves the stoichiometric conversion of the semiquinone to the hydroquinone, as is evident from the decrease in absorbance at 580 nm (0.5 mol of dithionite/mol of enzyme FAD). Exposure of the fully reduced enzyme to air causes a complete and rapid reoxidation of the flavin. A comparison of these observations on the reduction of $T. aquaticus$ PrxR by dithionite with similar titrations of PrxRs from other organisms is given in the discussion.

**Catalytic Properties**—PrxR from $T. aquaticus$ couples the oxidation of NADH to the reduction of several electron acceptors, including molecular oxygen (which it reduces to hydrogen peroxide), $\text{H}_2\text{O}_2$, DTNB, and Prx. The NADH oxidase activity is quite low unless exogenous FAD is added to mediate electron transfer from the enzyme-bound flavin (Table II (18)). The turnover numbers of the recombinant enzyme (mol of NADH$^{-1}$ - mol of enzyme FAD$^{-1}$) in the presence or absence of

![FIG. 4. SDS-PAGE and gel filtration analyses of $T. aquaticus$ Prx and PrxR. Top, SDS-PAGE gels after staining with Coomassie Blue. Panel A, mixture of native Prx and PrxR (lane 1) obtained following elution from AMP-Sepharose; panel B, native (lane 2) and recombinant (lane 3) PrxR; panel C, recombinant Prx (lane 4). The marker proteins are labeled (M) in each gel and were in order of decreasing size: bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.1 kDa), carbonic anhydrase (29.0 kDa), trypsinogen (24.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa). Bottom, determination of apparent molecular mass. The elution profile ($A_{280}$ versus retention time) of recombinant Prx (solid line) and recombinant PrxR (dashed line) was obtained by FPLC in a column of Superdex 200. A plot of $\log M_0$ of molecular mass versus retention time is also shown for the two enzymes (○) and for proteins used to calibrate the column (●): ferritin (450 kDa); β-amylase (200 kDa); bovine serum albumin (66.0 kDa); ovalbumin (45.0 kDa); cytochrome c (12.5 kDa), aprotinin (6.5 kDa).](image-url)
in assays with couples NADH oxidation to the reduction of hydrogen peroxide (18). Recombinant T. aquaticus Prx similarly couples NADH oxidation to the reduction of cumene hydroperoxide in the presence of Prx (AhpC) from S. typhimurium (18). Recombinant T. aquaticus PrxR similarly couples NADH oxidation to the reduction of hydrogen peroxide in assays with S. typhimurium Prx (Fig. 6; Table II). The corresponding activity with peroxiredoxin from T. aquaticus is much lower (Fig. 6). Thus, the turnover number for recombinant T. aquaticus PrxR in the presence of 5 μM S. typhimurium Prx is 1107 min⁻¹ at 25 °C, whereas it is greater than 80 times smaller when Prx from T. aquaticus replaces the S. typhimurium enzyme. Efforts to enhance the activity by pretreating Prx with dithiothreitol have failed. The turnover number for the mixture of the two T. aquaticus enzymes increases to 171 min⁻¹ when the assay temperature is 70 °C, the temperature at which the organism is grown. The overall activity at both temperatures depends on the concentrations of the two coupled enzymes. The rate at 25 °C increases linearly with concentration up to 144 nM PrxR when the assay is done with 5 μM Prx. It was not possible to study the effect of varying the concentration of Prx in the presence of a similarly high concentration of PrxR because the flavoprotein itself appears to have peroxidase activity, and this relatively high blank oxidation of NADH in the presence of H₂O₂ obscures the activity enhancement given by Prx. The turnover numbers for the peroxidase activity of PrxR alone are 0.034 s⁻¹ and 0.47 s⁻¹ at 25 °C and 70 °C, respectively. The peroxidase activity of PrxR in the presence of Prx is enhanced 1.3-fold by ammonium sulfate (apparent Kₘ = 11.4 mM at 25 °C), similar to the effect of ammonium sulfate on the activities of the homologous enzyme system from S. typhimurium (14). It is not known if this activation by ammonium sulfate is a specific one or a general ionic strength effect as observed in the A. xylanus system (44). It appears that in the case of the T. aquaticus enzyme the effect is on PrxR because the blank peroxidase activity of this enzyme is similarly enhanced by ammonium sulfate and with a similar activation constant (apparent Kₘ = 14.6 mM at 25 °C).

As noted above, T. aquaticus PrxR couples the oxidation of NADH to the reduction of cumene hydroperoxide in the presence of S. typhimurium Prx. This organic hydroperoxide appears to lead to inactivation of T. aquaticus Prx, because when cumene hydroperoxide replaces hydrogen peroxide in the com-

saturating FAD are similar to the corresponding turnover numbers of the native enzyme. However the specific activities determined for the native (37.0 units/mg) and recombinant (38.5 units/mg) forms of the enzyme in the standard assay used to monitor the purification of the enzyme (0.126 mM FAD) are about 50% greater than reported for an earlier preparation of native enzyme (24.9 units/mg (18)). The explanation for this difference is not known. The possibility that the discrepancy is due to the use of a different protein assay in the earlier work is ruled out by the observation in the present study that the two protein assays give identical results.

T. aquaticus PrxR also catalyzes the reduction of DTNB, a thiol-disulfide interchange activity that it shares with PrxR isolated from A. xylanus (43) and S. typhimurium (10). The kinetic constants determined for the recombinant enzyme are similar to those determined for native enzyme. However, the turnover number for the native enzyme determined under standard conditions (at 0.4 mM DTNB) is 26 times greater than was determined with an earlier preparation of enzyme (18), and again, the explanation for this difference is not known. Earlier work showed that native PrxR (NADH oxidase) from T. aquaticus couples NADH oxidation to the reduction of cumene hydroperoxide in the presence of Prx (AhpC) from S. typhimurium (18). Recombinant T. aquaticus PrxR similarly couples NADH oxidation to the reduction of hydrogen peroxide in assays with S. typhimurium Prx (Fig. 6; Table II). The corresponding activity with peroxiredoxin from T. aquaticus is much lower (Fig. 6). Thus, the turnover number for recombinant T. aquaticus PrxR in the presence of 5 μM S. typhimurium Prx is 1107 min⁻¹ at 25 °C, whereas it is greater than 80 times smaller when Prx from T. aquaticus replaces the S. typhimurium enzyme. Efforts to enhance the activity by pretreating Prx with dithiothreitol have failed. The turnover number for the mixture of the two T. aquaticus enzymes increases to 171 min⁻¹ when the assay temperature is 70 °C, the temperature at which the organism is grown. The overall activity at both temperatures depends on the concentrations of the two coupled enzymes. The rate at 25 °C increases linearly with concentration up to 144 nM PrxR when the assay is done with 5 μM Prx. It was not possible to study the effect of varying the concentration of Prx in the presence of a similarly high concentration of PrxR because the flavoprotein itself appears to have peroxidase activity, and this relatively high blank oxidation of NADH in the presence of H₂O₂ obscures the activity enhancement given by Prx. The turnover numbers for the peroxidase activity of PrxR alone are 0.034 s⁻¹ and 0.47 s⁻¹ at 25 °C and 70 °C, respectively. The peroxidase activity of PrxR in the presence of Prx is enhanced 1.3-fold by ammonium sulfate (apparent Kₘ = 11.4 mM at 25 °C), similar to the effect of ammonium sulfate on the activities of the homologous enzyme system from S. typhimurium (14). It is not known if this activation by ammonium sulfate is a specific one or a general ionic strength effect as observed in the A. xylanus system (44). It appears that in the case of the T. aquaticus enzyme the effect is on PrxR because the blank peroxidase activity of this enzyme is similarly enhanced by ammonium sulfate and with a similar activation constant (apparent Kₘ = 14.6 mM at 25 °C).

As noted above, T. aquaticus PrxR couples the oxidation of NADH to the reduction of cumene hydroperoxide in the presence of S. typhimurium Prx. This organic hydroperoxide appears to lead to inactivation of T. aquaticus Prx, because when cumene hydroperoxide replaces hydrogen peroxide in the com-

![Fig. 5. Reductive titration of recombinant T. aquaticus PrxR with dithionite ion.](image-url)

![Fig. 6. Peroxidase activity of T. aquaticus PrxR in the presence of Prx from T. aquaticus or S. typhimurium.](image-url)
plicate anaerobic assay with the two recombinant enzymes from *T. aquaticus*, the rate of NADH oxidation declines rapidly with time. A similar inactivation by peroxides has been reported for peroxiredoxin from yeast (45).

The recombinant form of the flavoenzyme PrxR shows high thermal stability, similar to that previously reported for the native enzyme (18). The stability at high temperature is enhanced by including FAD in the incubation mixture (Fig. 7). This suggests that the main cause of loss in activity at high temperature is dissociation of flavin from the enzyme and subsequent denaturation of the apoenzyme. The temperature stability of Prx has not been examined.

### DISCUSSION

We have shown that *T. aquaticus* contains a peroxiredoxin (Prx) and a corresponding reductase (PrxR) that allows the oxidation of NADH to be coupled to the reduction of hydrogen peroxide. Peroxiredoxins have been identified recently in a wide variety of organisms, and flavoenzymes that catalyze the reduction of Prxs are known to occur in certain other bacteria. This is the first time that the two genes for the enzyme system have been cloned and overexpressed from a thermophile and evidently is stabilized thermodynamically. It is probably more likely, however, is the possibility that the native enzyme titration would be distorted by oxygen contamination. Even more likely, however, is the possibility that the native enzyme that was used earlier contained either apo-PrxR and/or Prx. It is now known that it is difficult to completely separate Prx from PrxR when the two proteins are extracted from *T. aquaticus*. The presence of Prx and/or apo-PrxR in the titration of PrxR would increase the stoichiometry of reduction by contributing additional cystine disulfides to the overall reaction.

The stoichiometry of reduction of *T. aquaticus* PrxR by dithionite ion is similar to the stoichiometries reported for similar titrations of peroxiredoxin reductases (AhpF) from *A. xylosoxidans* (43) and *S. typhimurium* (10), as are the changes in the optical spectra in the various phases of reduction. In all cases, a high proportion of the flavin is converted to the semiquinone. However, the semiquinone is formed slowly in the titrations and evidently is stabilized thermodynamically. It is probably not important as an intermediate in catalysis (47).

In conclusion, the successful cloning, overexpression of the genes for *T. aquaticus* Prx and PrxR and the isolation of the two proteins in large amounts opens the way to detailed studies on their structures and catalytic mechanisms, including the use of site-directed mutagenesis to study the effects of replacing amino acids whose side chains may play important roles in the overall functions of the enzyme system.
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