Characterization of Glutathione Amide Reductase from Chromatium gracile

IDENTIFICATION OF A NOVEL THIOL PEROXIDASE (Prx/Grx) FUELED BY GLUTATHIONE AMIDE REDOX CYCLING

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Among the Chromatiaceae, the glutathione derivative γ-L-glutamyl-L-cysteinylglycine amide, or glutathione amide, was reported to be present in facultative aerobic as well as in strictly anaerobic species. The gene (garB) encoding the central enzyme in glutathione amide cycling, glutathione amide reductase (GAR), has been isolated from Chromatium gracile, and its genomic organization has been examined. The garB gene is immediately preceded by an open reading frame encoding a novel 27.5-kDa chimeric enzyme composed of one N-terminal peroxiredoxin-like domain followed by a glutaredoxin-like C terminus. The 27.5-kDa enzyme was established in vitro to be a glutathione amide-dependent peroxidase, being the first example of a prokaryotic low molecular mass thiol-dependent peroxidase. Amino acid sequence alignment of GAR with the functionally homologous peroxidase and trypanothione reductases emphasizes the conservation of the catalytically important redox-active disulfide and of regions involved in binding the FAD prosthetic group and the substrates glutathione amide disulfide and NADH. By establishing Michaelis constants of 97 and 13.2 μM for glutathione amide disulfide and NADH, respectively (in contrast to KM values of 6.9 mM for glutathione disulfide and 1.98 mM for NADPH), the exclusive substrate specificities of GAR have been documented. Specificity for the amidated dipeptide, to some extent, has to rely on the glutathione reductase (GR1)-dependent glutathione (GSH) redox cycle. Moreover, in eukaryotes, some reactive oxygen intermediates are detoxified directly by the action of glutathione peroxidases (6) and, to a lesser extent, of glutathione S-transferases (7). Members of another enzyme family of GSH-dependent thiol-disulfide oxidoreductases, designated thioltransferases or glutaredoxins (Grx), are believed to act as one of the primary defenders against mixed disulfides formed after oxidative damage to proteins (8). Besides these direct and indirect protection systems against the products of aerobic metabolism relying on GSH cycling, GSH itself also serves an indirect antioxidant function by protecting the amino acid cysteine against auto-oxidation (9). Only three groups of prokaryotes, the Gram-negative cyanobacteria and purple bacteria and some Gram-positive streptococci and enterococci (10, 11), produce GSH together with the recycling GR (12). Thus far, no significant GSH-dependent peroxidase activity has been reported for a GSH producing prokaryote. On the other hand, substantial thioltransferase activity is encountered indicating that in terms of oxygen shielding, GSH metabolism in prokaryotes does not serve a direct detoxification system for reactive oxygen intermediates but only maintains disulfides in the reduced state. Prokaryotic aerobes and pathogens require an array of antioxidant defense mechanisms to protect themselves against the reactive oxygen intermediates produced by the incomplete reduction of oxygen during respiration or by the antimicrobial response of the host phagocytes (13, 14). Detoxification of the freely diffusible hydrogen peroxide (H2O2), which in turn can be reduced further via the Fenton reaction to extremely reactive hydroxyl radicals, is completed by the action of catalases, heme- and manganese-containing peroxidases, and several members of the large multifunctional AhpC/TSA protein family, recently classified as peroxidoredoxins (Prx). To date, it seems that all bacterial Prx

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1 The abbreviations used are: GR, glutathione reductase; GSH, glutathione; Grx, glutaredoxin; Prx, peroxiredoxin; GASH, glutathione amide; GASSAG, glutathione amide disulfide; GAR, glutathione amide reductase; Prx/Grx, chimeric enzyme composed of one Prx and one Grx homologous domain; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Fmoc, (9-fluorenyl)methoxycarbonyl; OPfp, pre-activated pentafluorophenyl esters; HPLC, high pressure liquid chromatography; CV, column volumes; ORF, open reading frame.

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enzymes obtain the necessary reducing equivalents from the thioredoxin reducing system, because it was demonstrated recently that the flavoprotein component (AhpF) of the Salmonella typhimurium alkyl hydroperoxide reductase (AhpCF) system and bacterial thioredoxin reductase have very similar mechanistic properties (15). Apparently, GSH is never involved in bacterial Prx-reduction.

Chromatium species are anaerobic sulfur-oxidizing phototrophs that produce glutathione amide (GASH) (16), a GSH derivative modified at the terminal glycine. An original anaerobic function for this GASH metabolism was proposed by Pott and Dahl (17) and implies a possible involvement in the transfer of sulfide across the periplasmic membrane. When grown photoautotrophically on sulfide, GASH is present in its persulfide form (16), supporting the hypothesis that the periplasmically formed persulfide becomes transported to the cytoplasm, where the GASH-bound sulfide is released by the action of a heterodisulfide reductase. Chromatium species extracts do show glutathione amide disulfide (GASHG) reductase activity (16), and the involvement of GAR as the heterodisulfide reductase in the hypothesized sulfide transfer mechanism has to be considered because GSH persulfide reduction was established already for the bovine GR (18).

Here we present the isolation and successful expression in Escherichia coli of the Chromatium gracile garB gene, which has permitted the characterization of the C. gracile GAR enzyme. Further, we provide evidence for the existence of a novel Prx-containing peroxidase system, probably widespread among Gram-negative pathogens, that is fueled by the GAR-dependent redox cycling of the Chromatium-specific low molecular weight thiol GASH.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes were from New England Biolabs (Beverly, MA). T4 DNA ligase, the DIG DNA labeling kit, and the DIG luminescent detection kit were obtained from Roche Diagnostics. T7g DNA polymerase was from Amersham Pharmacia Biotech. Plasmid DNA was prepared on a 30-ml scale using the Qiagen (Crawley, UK) plasmid purification kit. The pGEM-T and pUC18 plasmids were from Promega (Madison, WI), and the pET11a plasmid was from Novagen (Madison, WI). DNA sequencing was performed using an ABI PRISM 377 automated sequencer (Applied Biosystems). All other biochemical reagents were purchased from Sigma-Aldrich. A Uvikon 943 double beam UV-visible spectrophotometer (Kontron Instruments, Watford, UK) was used for the spectroscopic measurements.

Strains and Media

C. gracile (DSM 1712) was grown on Pfennig's medium (19), supplemented with 1% NaCl, by anaerobic photosynthesis at a temperature of 30 °C. E. coli strains were grown on LB medium (Life Technologies, Inc.) supplemented with 100 μg/ml carbenicillin when necessary. Strain XL-1 Blue (New England Biolabs, Herefordshire, UK) was used as a recipient to detect α-complementation for pGEM-T derivatives on LB plates supplemented with 80 μg isopropyl-β-D-thiogalactoside and 32 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside. All expression plasmids were introduced into competent BL21(DE3) E. coli (Novagen).

N-terminal Amino Acid Sequence Determination

GAR was partially purified according to the method of Chang and Hurlbert (20). The partially purified enzyme sample was loaded onto an SDS-polyacrylamide gel, and after electrophoresis, the proteins were transferred onto a ProBlott membrane (Applied Biosystems) as described by Maniatis et al. (21). The membrane was stained with Coomassie Blue, and the protein band corresponding to the GAR enzyme was subjected to N-terminal sequence determination using a 477A pulsed liquid sequenator (Applied Biosystems). Sequencing reagents from the same sample. Forty-nine residues were identified covering a 51-amino acid N-terminal stretch, TQHFDLIAIGGSGGGLAVAEK-AAAFGKRVALIESKALGTXNVNXFPKVY. The same procedure was applied for the first seven amino acid residues of the partially purified recombinant PrxGrx.

SDS-PAGE

Protein samples were subjected to reducing SDS-polyacrylamide gel electrophoresis (22) and stained with Coomassie Blue or Silver (23). The total protein concentration was determined by the method of Bradford (24) using the Bio-Rad 500-0006 kit with bovine serum albumin as a standard.

DNA Techniques

C. gracile genomic DNA was isolated according to the cetyl-trimethyl-ammonium-bromide method described by Ausubel et al. (25). A 150-base pair fragment was obtained from C. gracile genomic DNA via PCR amplification using degenerate primers based on the N-terminal amino acid sequence of endogenous GAR (forward primer, 5′-ACICAR-CAYTTGYGAY-3′; reverse primer, 5′-CYTTTTTGGGACRCA-3′). This PCR fragment was ligated into the pGEM-T vector, and the resulting construct (pGEM-GAR) was verified by sequence analysis. The pGEM-GAR construct was used as a template for PCR-based synthesis of a digoxigenin-labeled probe using the DIG DNA labeling kit. Therefore, a perfect match primer pair was designed (forward primer, 5′-ACCCAG-CATTTCGACCTG-3′; reverse primer, 5′-CCTCTTGGGACGCGACG-3′). C. gracile subgenomic DNA fragments, generated by subjecting the genomic DNA to the action of various restriction endonucleases, were screened with the digoxigenin-labeled probe in Southern hybridization experiments. A single signal corresponding to a fragment of ~3.6 kilobases was obtained when the genomic DNA was initially cut with BamHI. A pUC18-subgenomic library of C. gracile DNA consisting of BamHI fragments between ~3.4 and 3.9 kilobases was screened by colony hybridization with the digoxigenin-labeled digonucleotide. The sequencing transforms were performed by the DNA sequencing detection kit according to the manufacturer’s instructions. Nucleotide sequence determination of one positive pUC18 derivative was initiated using the pUC18 universal primers M13F and M13R, and new primers were synthesized at ~450 nucleotide intervals based on the results of previous sequencing.

Synthesis of GASH and GASSAG

GSH was synthesized on the Advanced ChemTech 90 peptide synthesizer (Louisville, KY) using the N-(9-fluorenyl)methoxycarbonyl (Fmoc) strategy (26) on a polyamide resin developed for the synthesis of peptide amides (0, 52 mmol) (Rink Resin, Advanced ChemTech) (27). Fmoc-t-amino acids (3 eq = 1, 56 mmol) were introduced into the chain as pre-activated pentafluoroethyl esters (OPP) in the presence of N,N-diisopropylcarbodiimide (1 eq). The introduced residues were Fmoc-Gly-OPP, Fmoc-Cys(Trt)-OPP, and N-ac-butoxycarbonyl-Glu(OPP)-a-t-butyl. The efficiency of coupling was always checked by the Kaiser test. Deprotection of the Fmoc groups was carried out with 20% piperidine in N,N-dimethylformamide for 15 min. After washings, the peptide was liberated with a 95:5 (v/v) solution of trifluoroacetic acid/1,2-ethanedithiol for 90 min under nitrogen. After filtration, the trifluoroacetic acid was removed under vacuum. The peptide mixture in water was treated with diethyl ether (1:1) to eliminate scavengers. GASSAG was prepared according to the method described by Bartsch et al. (16) and purified by HPLC on a C18 reverse-phase column.

Overexpression of GAR and Prx/Grx

The garB gene was PCR-amplified, allowing the incorporation of NdeI (forward primer, 5′-GGGAATTCATATGACCCAGCATTTCG-3′) and NdeI/BamHI reverse primer, 5′-CAGGGATCCCTTAGGCGCTGGCGCGCTCC-3′. This PCR fragment was ligated into the pET11a expression plasmid pET11a. The resulting constructs (pET-GAR and pET-Prx/Grx), first verified by the DIG luminescent detection kit according to the manufacturer’s instructions, were then cut with NdeI/BamHI and cloned into the NdeI/BamHI site of the plasmid pBlueScript SK-+. The resulting plasmid (pBlueScript SK-+garB) was transformed into the competent E. coli strain DH5α. The colonies were purified by restriction analysis and sequenced. The correct sequencing was verified for all of the transformed colonies. The purified plasmids, pBlueScript SK-+garB, were then used for bacterial expression. The transformed bacteria were grown in LB medium containing 100 μg/ml carbenicillin, and the culture was incubated for 10 h. The culture was used to inoculate 4 liters of LB medium containing the antibiotic at a ratio of 10 ml/liter. When the A600 value of the culture reached the value 0.7–1.0, isopropl-

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β-D-thiogalactoside was added to a final concentration of 1 mM. Incubation was continued for 15 h at 37 °C, after which the cells were harvested by centrifugation (4000 x g) for 15 min, resuspended in 60 ml of sonication buffer (50 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA), and stored at −80 °C overnight. The cells were broken using a Branson sonicator with four 30-s bursts of 45 watts with 30-s intervals.

**Protein Purification**

GAR—The crude extract from a GAR-producing culture was clarified by centrifugation at 15,000 x g for 30 min at 4 °C and fractionated with solid (NH₄)₂SO₄. The 20–60% saturation precipitate was dissolved in 20 ml of buffer A (50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 x (NH₄)₂SO₄). The enzyme solution (2 ml/run) was applied onto a butyl-Sepharose packed HR 16/10 column (Amersham Pharmacia Biotech) equilibrated with buffer A. After loading, the resin was washed with 7 column volumes (CV) of the same buffer. The elution of GAR occurred with a decreasing step gradient of (NH₄)₂SO₄ (step 1, 1000–450 mM in 8 CV; step 2, 450–375 mM in 10 CV; step 3, 375–0 mM in 1 CV) at a rate of 2 ml/min. The enzyme eluted during step 3 and the GAR-containing fractions of 10 runs were pooled, concentrated using a Microcon centrifugal filter (Millipore, USA) and dialyzed against 20 mM Tris-HCl, pH 7.0. The concentrate was further purified on the ResourceQ anion exchanger (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris-HCl, pH 7.0. The yellow enzyme was eluted with a 30-CV linear gradient from 0 to 1 x NaCl in 20 mM Tris-HCl, pH 7.0, at a rate of 4 ml/min. The protein eluted at ~0.49 M NaCl. Finally, the pooled fractions were concentrated and loaded onto a HiLoad 16/60 Sephacryl 75 gel-sizing column (Amersham Pharmacia Biotech) previously equilibrated with 10 mM Tris-HCl, pH 7.0. Elution occurred up-flow with 10 mM Tris-HCl, pH 7.0, at a flow rate of 2 ml/min. SDS-PAGE analysis of the pooled yellow-colored fractions indicated that GAR was purified to homogeneity. The gel-sizing protocol was also applied for native molecular mass determination. Molecular mass standards were derived from a gel filtration calibration kit (Amersham Pharmacia Biotech) containing ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), and Dextran 2000 (2000 kDa).

The absorption coefficient of 11.0 mM⁻¹ cm⁻¹ was determined spectroscopically at 461 nm by using an absorption of 11.0 μM⁻¹ cm⁻¹/active site.

**Prx/Grx—**The cell debris of a Prx/Grx-containing crude extract was precipitated by centrifugation at 15,000 x g for 30 min at 4 °C, and the supernatant was fractionated with solid (NH₄)₂SO₄. The 30–70% saturation precipitate was dissolved in 20 ml of buffer B (50 mM sodium phosphate, pH 6.4, containing 150 mM NaCl, TOTA, and 1.5 M (NH₄)₂SO₄). The enzyme solution (2 ml/run) was then loaded onto an octyl-Sepharose packed HR 16/10 column (Amersham Pharmacia Biotech) equilibrated with buffer B. After loading, the resin was washed with the same buffer for 10 CV. The protein was eluted with a decreasing step gradient of (NH₄)₂SO₄ (step 1, 1500–450 mM in 8 CV; step 2, 450–375 mM in 15 CV; step 3, 375–0 mM in 1 CV) at a rate of 2 ml/min. Fractions containing Prx/Grx, as assessed by activity measurements and SDS-PAGE analysis, were pooled during step 3, concentrated, and dialyzed against buffer C (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) of the same buffer. The dialyzed protein solution was applied onto a ResourceQ column pre-equilibrated with buffer C. The column was washed with 5 CV of buffer C, and the protein was eluted with a 25-CV linear salt gradient (0.0–1.0 M NaCl in buffer C) at a flow rate of 3.5 ml/min. Prx/Grx eluted at ~0.22 M NaCl. SDS-PAGE analysis indicated the presence of the 27.5-kDa Prx/Grx—enzyme complex with a few containing protein bands.

**Mass Determination**

Mass spectral analysis of the proteins was performed using nanospray ionization on a hybrid quadrupole time-of-flight mass spectrometer (Micromass, Whytenshawe, UK). The protein sample was diluted to ~5 pmol/μl in acetonitrile/water/formic acid (1:1:0.01). 3 μl of the dilution was loaded in a coated borosilicate needle (Protana, Denmark). The needle was placed into the quadrupole time-of-flight source, and after breaking the tip, a voltage of 1350 V was applied. To determine the native molecular weight and the type of quaternary structure, the solvent was replaced by water. The source temperature was 30 °C in all cases. The mass spectrometer was calibrated independently using NaCl. Mass spectral identification of the prostatic flavin of GAR was performed under the same conditions as described for the subunit mass determination, except the negative ion mode of analysis was applied.

**Enzyme Assay**

GAR—GAR activity was measured by two methods. For all determinations, a GASSAG GASSAG-dependent NADH oxidation, again monitored at 340 nm. Unless otherwise stated, the reconstitution assay contained 0.4 units of GAR, 150 μM NADH, 500 μM GASH, 50 μM partially purified Prx/Grx, and 100 μM H₂O₂ or small alkyl hydroperoxides in 0.5 ml of 125 mM potassium phosphate buffer, pH 7.9, and 0.1 mM EDTA. The reaction was started with the addition of the hydroperoxide. No blank rate of NADH oxidation was observed during the reconstitution assay devoid of hydroperoxide. In the specificity study, GASH, GAR, and NADH were replaced by yeast GR (0.4 units), GSH (500 μM), and NADPH (150 μM), respectively.

**RESULTS**

**Nucleotide and Amino Acid Sequence Analysis**

The isolated garB gene-containing BamHI fragment (Fig. 1) consists of 2519 base pairs and comprises three open reading frames (ORFs): garA, garB, and an ORF encoding a polyepitope that is 67% identical to a hypothetical E. coli protein. The GAR enzyme-encoding garB gene, 1392 base pairs long, translates to a peptide of 463 amino acids. However, in accordance with the situation for the E. coli (29) and Plasmodium falciparum GR enzymes (30) and for the Trypanosoma congolense trypanothione reductase (31), the N-terminal amino acid sequence analysis of the endogenous as well as of the recombinant GAR revealed that the initiator methionine is post-translationally deleted. Comparison with E. coli GR (Fig. 2 and Crichtida fasciculata trypano-thione reductase (32) reveals 49 and 34.5% amino acid sequence identity, respectively, and emphasizes the conservation of the motifs essential for binding of the prosthetic group and substrates in the C. gracile enzyme.

The garA and garB genes are separated by 185 base pairs and are transcribed in the same direction. A computer search for putative promoter sequences points to the cotranscription of the two ORFs. The enzyme deduced from garA comprises 247 amino acids and seems to be chimeric, having an N-terminal part that is significantly homologous to the Prx family enzymes (33, 34) and a C-terminal domain that is highly related to Prx (35). Computer alignment of the entire Brassica rape CpxII, a Prx peroxidase that has been shown to catalyze the reduction of hydrogen peroxide with the use of electrons from the thioredoxin system (33), with amino acid residues 1–163 of the garA deduced chimeric protein reveals 39% identity and striking homology around the N-terminal cysteine residue, which is believed to be the site of oxidation by peroxides (36). A similar alignment of the E. coli Grx3 (5) with amino acid R171 suggests that the Grx typical redox-active Cys-Pro-X-Cys motif and reveals 62% homology.

The genomes of the Haemophilus species, Yersinia pestis, Pasteurella multocida, Vibrio cholerae, Bordetella pertussis, and Thiomicrobium ferroxidans, also encode a homologue of the Prx/Grx coding sequence along with a glutathione reductase gene, but only the latter organism has both genes being organized in an operon as is the case in C. gracile.

**Characterization of Recombinant GAR**

**Physical and Spectral Characterization**—Recombinant GAR was overexpressed and purified to homogeneity (Fig. 3) as described under “Experimental Procedures.” Because E. coli strain BL21(DE3) contains an active glutathione reductase gene, the possibility of contamination of the vector-encoded GAR with the chromosomally encoded GR had to be considered.
However, the two disulfide reductases were well resolved by chromatography, because neither GR activity nor a GR-derived additional mass spectral peak of 48,641 Da was detected after the analysis of finally purified GAR. A subunit mass of 49,030 Da obtained using nanospray ionization on a quadrupole time-of-flight mass spectrometer agrees well with the value calculated from the deduced amino acid sequence, 49,159 Da, minus 131 Da corresponding to the N-terminal methionine residue being absent in the native enzyme. A native molecular mass of 98,000 Da was obtained from gel filtration chromatography and mass spectrometric analysis, which is consistent with a dimeric quaternary structure, a characteristic feature of the glutathione reductase family. The strength of the GAR dimer is significantly increased in the presence of copper ions, because boiling and SDS treatment failed to disrupt the GAR dimer conformation after the addition of CuCl2 at micromolar levels (Fig. 3). We established that the gradual increase in copper-mediated dimer strength concurs with the gradual decrease of GAR activity observed when comparable CuCl2 concentrations were added to the reaction mixture (data not shown). Mn2+, Ni2+, Zn2+, Hg2+, and Ca2+ ions were also tested in their capacity to capture the dimer state, but they all failed to do so. Copper is, after mercury, the most effective metal ion inhibitor of members of the glutathione reductase family (20, 37). The visible absorption spectrum of oxidized GAR shows maxima at 273, 378, and 461 nm, with a shoulder at 481 nm, indicating the presence of a flavin cofactor. The spectral ratio $A_{280}/A_{461}$ of the homogeneous enzyme was determined to be 7.2 and is similar to other glutathione reductases (7.2 and 7.1 for the *E. coli* (29) and *Enterococcus faecalis* (11) enzymes, respectively). To identify the flavin prosthetic group, the flavin was liberated by thermal denaturation of the protein at 100 °C for 20 min. The resulting free flavin showed absorption maxima at 370 and 450 nm, and the ratio of $A_{461}$ to $A_{370}$ was determined to be 1.25, which is similar to other glutathione reductases. 

FIG. 1. Nucleotide sequence of the *C. gracile* subgenomic BamHI fragment containing the genes *garA* and *garB*, encoding Prx/Grx and GAR, respectively, together with a third ORF, encoding a novel Prx/Grx hybrid peroxidase. The amino acid sequences shown are deduced from the sequenced nucleotides with the exception of the boxed residues of the GAR and Prx/Grx enzyme, which were identified by N-terminal protein sequence analysis. The boxed sequences shown in capitals are the *Bam* HI sites.
the enzyme-bound flavin to $A_{450}$ of the free flavin yielded an absorption coefficient for the GAR enzyme of $e_{450} = 11.0 \text{ mm}^{-1} \text{ cm}^{-1}$ (S.D. = 0.1 mm$^{-1}$ cm$^{-1}$; $n = 4$). The liberated flavin was purified by HPLC and identified by negative ion mode mass spectral analysis to be FAD. Upon anaerobic titration with NADH, the spectrum of the enzyme exhibits decreases in absorbance at 461 nm and increases at 530 nm (Fig. 4), demonstrating the formation of a charge-transfer complex between one of the newly reduced active site thiols and the oxidized FAD.

Kinetic Characterization—C. gracile GAR exhibits a high specificity for the substrate GASSAG and the reductant NADH, both of which display Michaelis-Menten saturation kinetics (data not shown). The $K_m$ for GASSAG with 100 $\mu$M NADH was 97 ± 5 $\mu$M, and a turnover number of 14,981 ± 680 $\mu$mol min$^{-1}$ FAD was calculated, yielding a $k_{cat}/K_m$ of $2.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. The apparent $K_m$ and $k_{cat}$ values for NADH with 500 $\mu$M GASSAG were 13.2 ± 1.8 $\mu$M and 11,210 ± 90 $\mu$mol min$^{-1}$ FAD, respectively, yielding a $k_{cat}/K_m$ of $14.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. The observed poor activity of the reductase with either glutathione disulfide or NADPH are, in the case of the former substrate, mostly because of the weak binding to the active site of GAR ($K_m = 6.9 ± 0.2 $mm, $k_{cat} = 8759 ± 65 $\mu$mol min$^{-1}$ FAD), whereas in the case of the dinucleotide, binding as well as catalysis are seriously diminished (apparent $K_m = 1.98 ± 0.1$ mm and apparent $k_{cat} = 805 ± 24 $\mu$mol min$^{-1}$ FAD).

Reconstitution of a GASH-dependent Peroxidase System—Even though the amino acid sequence of the C. gracile Prx/Grx chimeric enzyme has no homology with recently characterized eukaryotic non-selenium glutathione peroxidases (38,
and no GSH-dependent peroxidase activity was observed in Chromatium vinosum extracts (40), we tested the possibility that the C. gracile GASH metabolism could serve a direct antioxidant function by neutralizing peroxides. Many Prx-type enzymes have indeed been proven to be potent peroxide reducers through the use of electrons coming from different sources (33, 34, 41, 42), and evidently no GSH-dependent peroxidase activity could have been observed in GASH-producing Chromatium species. Therefore, recombinant Prx/Grx was partially purified (see “Experimental Procedures”), after which the first seven amino acid residues were verified by N-terminal sequence analysis. A polypeptide with a molecular mass of 27,500 Da was visible upon SDS-PAGE analysis (Fig. 3), matching the value of 27,426 Da calculated from the deduced amino acid sequence. The hydroperoxide-dependent Prx/Grx-catalyzed oxidation of GASH was established in vitro, and as to be expected, the reaction is fueled by NADH via GASH redox cycling (Fig. 5). Nevertheless, the GSH-reducing system also seems to be an effective electron donor (Fig. 5, curves 4, 7, and 9). The Prx/Grx enzyme displays a similar activity with hydrogen peroxide (Fig. 5b) and small alkyl hydroperoxides (Fig. 5, a and c), indicating broad substrate specificity.

**DISCUSSION**

GASH, \(\gamma\)-L-glutamyl-L-cysteinyglycine amide, is a recently discovered derivative of GSH that has been found in strictly anaerobic as well as in aerotolerant Chromatium species (16). Extracts of Chromatium species were found to contain an enzyme activity capable of reducing oxidized GASH (16). The work reported here demonstrates that the activity is carried out by a new member of the class I flavoprotein disulfide reductase family of oxidoreductases, which includes GR (43, 44), trypanothione reductase (45), mycothione reductase (46), \(\gamma\)-glutamylcysteine reductase (9), mercuric reductase (47, 48), and lipoamide dehydrogenase (49). We could establish numerous structural and spectral homologies between the C. gracile GAR and glutathione reductases (50), making it clear that GAR is a new GR homologue. However, in addition to the altered substrate specificity of GAR, another striking difference lies in the fact that GAR displays a strong preference for NADH as the electron donor. Both specificity alterations are demonstrated by the summary of the kinetic parameters given in Table I. The table further illustrates that the C. gracile GAR and the human GR share comparable kinetic parameters, taking the preferred coenzymes and substrates into consideration. This can be demonstrated additionally by comparing the ratios of the specificity constants for the preferred substrates to those for the biologically irrelevant substrates. For the C. gracile enzyme, the apparent \(k_{cat}/K_m(\text{NADH})/k_{cat}/K_m(\text{NADPH}) = 2088\) and \(k_{cat}/K_m(\text{GASSAG})/k_{cat}/K_m(\text{glutathione disulfide}) = 122\), which are similar to the inverse ratios of the human enzyme (2158 and 87.7, respectively).
Three-dimensional structure analysis of the GR of human erythrocytes provided a detailed knowledge of the structural features in the active site that impart the specificity for the coenzyme (NADPH) and the substrate (glutathione disulfide) (51). Sequence alignment shows replacements disclosing in part the preference of GAR and GASSAG (Fig. 2). Seventeen of twenty-one active site substrate interacting residues, direct or solvent-based, are either identical or similar to those of the human enzyme. Three of the four nonconserved residues interact with the C-terminal glycines of the substrate. Of particular interest is Arg-37, which forms a salt bridge with the first substrate glycine carboxylate in the human GR enzyme and is substituted by Glu-21 in GAR. Comparison with the currently characterized GR homologues reveals that the enzyme of the trypanosomatids, which also catalyzes the reduction of a GSH derivative having a C-terminal amidation (N^2,N^5-bis(glutathionyl)spermidine), displays a negative charge in this region as well. This indicates that the Chromatium enzyme may be the only bacterial enzyme found so far that is specific for GASSAG (cf. for many enzymes in the family, the substrate specificity has not yet been examined).

In most NADH-dependent redox enzymes, the functional domain primarily responsible for binding the dinucleotide forms the take of a βββ-unit (52) with a highly conserved Gly-X-Gly-X-Gly sequence motif between the first β-strand and the succeeding α-helix (53). In NADH-binding domains the third glycine residue is often replaced by alanine (54). Furthermore, the negatively charged 2'-phosphate group of the AMP moiety of NADPH rests against a cushion provided by positively charged side chains. In the region corresponding to the βββ-unit in the GAR sequence, the complete Gly-X-Gly-X-Gly sequence motif is observed, and in addition, all three basic residues (Arg-218, His-219, and Arg-224) interacting in the human GR enzyme with the NADPH 2'-phosphate group are replaced (Leu-197, Glu-198, and Phe-203, respectively). The negative charge caused by Glu-198 is likely to be the primary cause of the change in reductant specificity. No other GR has a negative charge in this region, and therefore no other GR is thought to preferentially utilize NADH.

NADH-dependent enzymes are almost exclusively involved in the oxidative degradations that yield ATP, whereas NADPH-dependent enzymes, with few exceptions, are confined to the reactions of reductive biosynthesis. Therefore, the proposed anaerobic function for the Chromatium species-specific GASH metabolism as a sulfide carrier necessary for cytoplasmic sulfide oxidation (17) looks attractive. By analyzing the genomic organization of the garB gene encoding the crucial enzyme in GASH metabolism, we aimed to broaden our understanding with regard to the proposed sulfide carrier system. We found that the gene is immediately preceded by an ORF (which we termed garA because of the established cooperation between the garA and garB gene products) encoding a 27.5-kDa chimeric enzyme, clearly assembled from two enzyme entities separated by a seven-amino acid stretch containing three proline residues. The N-terminal component is strikingly homologous to Prx enzyme family members found in yeast, plants, and animals already characterized to reduce peroxides in a thioredoxin-dependent manner (33, 34, 55), whereas the second com-
ponent is homologous to glutaredoxins, which are known to reduce diverse disulfide bonds in a coupled system with GSH, NADPH, and GR. Based on the number of conserved cysteine residues and the properties revealed by immunoblot analysis, the Prx proteins can be subdivided into the subfamilies of the 1Cys-Prx and 2Cys-Prx proteins (56). Phylogenetic analysis, however, indicates that the Prx-like component of the *C. gracile* Prx/Grx peroxidase together with its homologues constitute a separate Prx type (57). We produced the recombinant 27.5-kDa Prx/Grx chimeric enzyme in *E. coli* and showed that it possesses peroxidase activity in reconstitution experiments using hydroperoxides, GASH, GAR, and NADH. Differing from the assumption that GSH-dependent hydroperoxide metabolism is catalyzed by a single enzymatic entity (6), we have demonstrated that the *C. gracile*-specific GASH metabolism (or the physiological irrelevant GSH metabolism) can deliver electrons to a peroxidase, which is most likely assembled from two enzymatic entities, and is capable of reducing H$_2$O$_2$ and small alkyl hydroperoxides at comparable rates.

These data, which show that the *C. gracile* Prx reducing electrons are Grx-derived (whereas in plants and animals those electrons come from the thioredoxin system), are additional support for the observation that thioredoxin and Grx systems can partially distribute for each other in vivo by reducing the same targets.

In conclusion, looking for a primordial anaerobic function for glutathione (amide) metabolism in the ancient phototrophic bacterium *C. gracile* (41) resulted in the identification of a unique cascade of oxidoreductase components that catalyzes GASH-mediated hydroperoxide metabolism. The possibility that the distribution of the Prx/Grx enzyme among *Chromatium* species determines to a certain extent the oxygen sensitivity of this bacterial family, containing strictly anaerobic as well as aerotolerant representatives, is currently a subject of study. Overall, next to the unique cascade of oxidoreductase components that uses the electrons provided by the redox capacity of trypanothione to finally reduce hydroperoxides (33), the *C. gracile* GASH-dependent peroxidase system illustrated here is the second low molecular weight thi-old-dependent alternative for the well documented members of the eukaryotic-specific (seleno-cysteine-containing) GSH peroxidase family. Because we have found homologues of the Prx/Grx peroxidase in the translated genomes of several Gram-negative pathogens, which are supposed to synthesize GSH, Prx/Grx is assumed to be a member of a novel enzyme family crucial for prokaryotic GSH-dependent direct protection against reactive oxygen intermediates.

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Characterization of Glutathione Amide Reductase from *Chromatium gracile*:
IDENTIFICATION OF A NOVEL THIOL PEROXIDASE (Prx/Grx) FUELED BY GLUTATHIONE AMIDE REDOX CYCLING

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