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The Thioredoxin System of *Helicobacter pylori**

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This paper describes the purification of thioredoxin reductase (TR) and the characterization, purification, and cloning of thioredoxin (Trx) from *Helicobacter pylori*. Purification, amino acid sequence analysis, and molecular cloning of the gene encoding thioredoxin revealed that it is a 12-kDa protein which possesses the conserved redox active motif CGPC. The gene encoding Trx was amplified by polymerase chain reaction and inserted into a pET expression vector and used to transform *Escherichia coli*. Trx was overexpressed by induction with isopropyl-1-thio-β-D-galactopyranoside as a decahistidine fusion protein and was recovered from the cytoplasm as a soluble and active protein. The redox activity of this protein was characterized using several mammalian proteins of different architecture but all containing disulfide bonds. *H. pylori* thioredoxin efficiently reduced insulin, human immunoglobulins (IgG/IgA/sIgA), and soluble mucin. Subcellular fractionation analysis of *H. pylori* revealed that thioredoxin was associated largely with the cytoplasm and inner membrane fractions of the cell in addition to being recovered in the phosphate-buffered saline-soluble fraction of freshly harvested cells. *H. pylori* TR was purified to homogeneity by chromatography on DEAE-52, Cibacron blue 3GA, and 2′,5′-ADP-agarose. Gel filtration revealed that the native TR had a molecular mass of 70 kDa which represented a homodimer composed of two 35-kDa subunits, as determined by SDS-polyacrylamide gel electrophoresis. *H. pylori* TR (NADPH-dependent) efficiently catalyzed the reduction of 5,5′-dithiobis(nitrobenzoic acid) in the presence of either native or recombinant *H. pylori* Trx. *H. pylori* Trx behaved also as a stress response element as broth grown bacteria secreted Trx in response to chemical, biological, and environmental stresses. These observations suggest that Trx may conceivably assist *H. pylori* in the process of colonization by inducing focal disruption of the oligomeric structure of mucin while rendering host antibody inactive through catalytic reduction.

Redox control of a broad range of biochemical and immunological processes is now well documented to exercise a pivotal role in the cellular activity of both eukaryotes and prokaryotes. To date the redox properties of *Helicobacter pylori* have received little attention. Accumulating evidence indicates that the redox status of cells controls various cellular functions including cellular activation and proliferation in addition to growth inhibition and apoptosis (for reviews, see Refs. 1–5). Cellular redox status is maintained by intracellular redox-regulating molecules, including thioredoxin (Trx),[1] glutaredoxin, and protein disulfide isomerase, which catalyze the formation and reduction of disulfide bonds in proteins.

The redox protein Trx and the associated enzyme thioredoxin reductase (TR) constitute a thiol-dependent reduction-oxidation system that can catalyze the reduction of certain proteins by NADPH, usually with high selectivity (1). In anaerobic bacteria, the generation of low redox potential reductants, such as Trx, can be used to assist electron flow to specific substrates. The capacity with which a given Trx can participate in this process is governed largely by the redox potential of the molecule. This in turn is directly associated with the nature of the amino acid residues flanked by 2 redox active vicinal cysteines in the active site. These cysteine residues participate in various redox reactions resulting in the catalysis of dithiol-disulfide exchange reactions. This C*XX*C motif has been termed elegantly as a molecular rheostat (6), whereby the resulting family of thiol-disulfide oxidoreductases vary greatly in their redox potential and therefore their ability to assist electron flow. Thioredoxin reductase catalyzes the reduction of oxidized thioredoxin (Trx-S_2) by NADPH, and reduced thioredoxin (Trx-$(SH)_2$) is the disulfide reductase. Typically, enzymes of this family contain 2 identical protein subunits, each subunit containing one redox-active disulfide, 1 mol of FAD per subunit, and conserved FAD and NAD(P)H binding motifs. The flavin moiety mediates the transfer of reducing equivalents from NADPH to the disulfide bond of thioredoxin.

We sought to identify and characterize processes in the gastric pathogen *H. pylori* which were susceptible to redox regulation, with an overall view to exploring the effects of the bacterium's redox environment on pathogenic mechanisms in addition to studying the effects of host factors on the redox activity of the bacterium. Initially we focused on members of the thioredoxin superfamily and specifically the Trx system. Here we describe the purification, overexpression, and characterization of the Trx system from *H. pylori*.

EXPERIMENTAL PROCEDURES

Materials—2′,5′-ADP-agarose, Cibacron blue 3GA, iminodiacetic acid-Sepharose 6B, ρ-aminobenzamidine-agarose, bovine insulin, bovine submaxillary gland mucin, DTT (1,4-dithio-DL-threitol) and *Escherichia coli* thioredoxin, and anti-*E. coli* thioredoxin were obtained from Sigma,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AE000594.

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[1] The abbreviations used are: Trx, thioredoxin; TR, thioredoxin reductase; 2′,5′-ADP-agarose, adenosine 2′,5′-bisphosphate linked to agarose through a 6-aminohexyl group; Trx-S2 and Trx-(SH)2, oxidized and reduced thioredoxin, respectively; DTT, 1,4-dithio-DL-threitol; TR, thioredoxin reductase; redox, reduction/oxidation; Ig, immunoglobulin; H_2L_2, intact Ig; H_2L, partially reduced Ig HL, heavy-light chain monomer; H, reduced heavy chain; L, reduced light chain; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; SC, secretory component.

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Poole, Dorset, United Kingdom. Sephacryl S-300 was obtained from Amersham Pharmacia Biotech. Isopropyl-β-D-thiogalactoside, NADPH, $NADP^+$, and NADH were obtained from Roche Molecular Biochemicals, Bell Lane, Lewes, East Sussex, U.K. DEAE-52 was purchased from Whatman (Maidstone, UK). Factor Xa was purchased from New England Biolabs, Hertfordshire, U.K. Polyclonal human IgG, secretory IgA, and IgA_1 were obtained from Calbiochem, Beeston, Nottingham, U.K. Anti-IgG subclass antibodies (anti-IgG_1, IgG_2, IgG_3, IgG_4) were purchased from The Binding Site Ltd., Birmingham, U.K. Anti-IgA and anti-secretory component antibodies were from Dako Ltd. All buffer reagents for SDS-PAGE were prepared in deionized water (Elga Prima reverse osmosis; water quality 1.2 μS/cm). All other chemicals were of analytical reagent grade.

Western Blotting and SDS-PAGE—Discontinuous SDS-PAGE was performed essentially as described previously (7). Proteins from SDS-PAGE gels were electroblotted (0.9 mA/cm^2 for 1 h) to polyvinylidene difluoride membrane (Gelman) using a semi-dry blotting apparatus (Amersham Pharmacia Biotech), essentially as described by Towbin *et al*. (8). Immunoblots were processed and developed by enhanced chemiluminescence as described previously (9). For NH_2-terminal sequencing the protein was electroblotted to ProBlott and stained briefly with freshly prepared Amido Black. Two-dimensional SDS-PAGE was performed essentially as described (54). Briefly, pellets of cells (~100 μg of protein) obtained from broth cultures of *H. pylori* were resuspended in isoelectric focussing (IEF) sample buffer and subjected to isoelectric focussing for 16 h at 300 V and for an additional 1 h 15 min at 800 V. Following IEF, the gels were equilibrated in SDS-PAGE sample buffer for 30 min prior to electrophoresis in the second dimension on 15% acrylamide gels. Broad range carrier ampholytes (pH 3–10) were used in the IEF gels.

Protein Measurements—Protein was measured by the method of Markwell *et al*. (10) with bovine serum albumin as the protein standard.

Bacterial Strain and Growth Conditions—The reference strain of *H. pylori* used in this study (NCTC 11638, $VacA^+$ and $CagA^+$) was obtained from the National Collection of Type Cultures, Public Health Laboratory, London, U.K. All components for *H. pylori* culture media were obtained from Oxoid, Unipath Ltd., Basingstoke, Hampshire, U.K. *H. pylori* was grown under microaerobic conditions (Oxoid Campylobacter system, 5% O_2, 10% CO_2) for 4 days on 7% lysed horse blood Columbia agar at 37 °C. Cells were harvested into ice-cold phosphate-buffered saline (pH 7.5). The cells were washed twice by centrifugation (10,000 × *g*, 5 min, 4 °C) in the appropriate buffer before use. Liquid cultures of *H. pylori* were grown in broth medium (10 ml) containing brain heart infusion, horse serum (5%, v/v), and yeast extract (0.25%, w/v) as described previously (55). The broth was supplemented with vancomycin (6 mg/liter), nalidixic acid (20 mg/liter), and amphotericin B (4 mg/liter) and incubated for 2 h at 37 °C in 5% CO_2. The flasks were then sealed and incubated with constant agitation (120 rpm) for a further 72 h at 37 °C. Cultures were examined by a rapid urease test (phenol red) and by subculture to solid media (Columbia blood agar and Brain Heart infusion agar) with appropriate incubation to confirm the identity and purity of the broth cultures. In some experiments bovine anti-*H. pylori* IgG (0.1 mg/ml) was added to the broth culture. Samples (0.5 ml) of the broth culture were taken before and at various periods of time after the addition of antibody. *H. pylori* was removed by centrifugation (10,000 × *g*, 3 min) and both the pellet of cells and the supernatant were retained and stored at −25 °C until required for analysis.

Purification of Thioredoxin Reductase (TR)—Agar-grown *H. pylori* was suspended in buffer A (20 mM Tris-HCl, pH 7.5) and subjected to sonication (4 × 1 min bursts) on ice using a Branson sonifier 450. After centrifugation to remove intact cells and cellular debris (12,000 × *g*, 10 min, 4 °C) the resulting supernatant was applied to a DEAE-cellulose column (3.5 × 16 cm) equilibrated in buffer A. Thioredoxin reductase activity was eluted with a gradient (300 ml) of KCl (0–0.35 M) in buffer A. Active fractions were pooled, dialyzed against buffer B (50 mM Tris-HCl, pH 7.5), and applied to a Cibacron blue 3GA column (1 × 3 cm). TR was eluted with a gradient of KCl (0–0.4 M). Active fractions were pooled, dialyzed against buffer B, and applied to a small 2′,5′-ADP-agarose column (1 ml). Thioredoxin reductase was eluted upon application of 0.2 M KCl. The ion exchange and dye affinity chromatography steps were performed at room temperature and the ADP-Sepharose step was done at 4 °C.

Gel Filtration Chromatography—A sonicate of *H. pylori* was prepared as described above and 0.5 ml (~10 mg of protein/ml) of the material was applied to a column (diameter 1.5 cm; height 29.7 cm) of Sephacryl S-300 superfine (Amersham Pharmacia Biotech) equilibrated with phosphate-buffered saline (pH 7.5) containing NaN_3 (0.02%, w/v). The protein was eluted with this same buffer (8.5 cm/h) and the collected fractions were assayed for both TR activity and total protein. The column was first calibrated with proteins of known molecular size (Amersham Pharmacia Biotech). Gel filtration over Sephadex G-50 (Amersham Pharmacia Biotech) was performed also in PBS.

Measurement of Thioredoxin Reductase Activity—Thioredoxin reductase activity was assayed at 25 °C in 0.1 M potassium phosphate buffer (pH 7.5) containing EDTA (1 mM), 5,5′-dithiobis(2-nitrobenzoic acid) (5 mM), and NADPH (0.2 mM) in a final volume of 1.0 ml. The reaction was initiated by the addition of enzyme and the progress of the reaction was monitored by the increase in absorbance at 412 nm in a Pye Unicam 5625 spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH/min at 25 °C (pH 7.5). Activity was calculated as micromole of NADPH oxidized/min in accordance with the relationship $\Delta A_{412}/(13.6 \times 2)$. Thioredoxin reductase activity was assayed also using a minor modification of the insulin reduction assay (11). The reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.0) containing EDTA (1 mM), insulin (0.1 mg/ml), NADPH (0.2 mM), and *H. pylori* histidine-tagged Trx (2 μM) in a final volume of 1 ml. The reaction was initiated by the addition the enzyme to the mixture at 25 °C and the oxidation of NADPH was monitored at 340 nm. The amount of NADPH oxidized was determined from the relationship $\Delta A_{340}/6.2$.

Purification of Native H. pylori Trx—Thioredoxin was purified by a combination of ion exchange chromatography on DEAE cellulose and gel filtration over Sephadex G-50. Fractions containing Trx were identified using the spectrophotometric insulin reduction assay (11).

Fluorescence Spectroscopy—Fluorescence emission spectra of Trx were determined with a Jasco FP 750 spectrofluorimeter by excitation at 280 nm and emission was recorded from 290 to 390 nm. All measurements were performed in Tris-HCl (50 mM, pH 7.3) containing EDTA (1 mM) and Trx (15 μg/ml) at 25 °C. To prevent oxidation of Trx, all solutions were sparged with N_2. Complete reduction of Trx was achieved by the addition of DTT (1 mM).

Expression and Purification of Recombinant H. pylori Trx—Transformants of *E. coli* BL21(DE3)pLysS with plasmid pET-16b (Novagen) containing the *Trx* gene (HP 824) were grown at 37 °C in LB broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml). *H. pylori* Trx was expressed as an NH_2-terminal decahistidine fusion protein in *E. coli*. The gene coding for Trx was amplified by polymerase chain reaction using Expand™ (Roche Molecular Biochemicals), using the amplification conditions recommended by the manufacturer. Under these conditions a single product was obtained and this was cloned into the expression plasmid via the *Bam*HI and *Nde*I restriction sites. The following primers were used: forward primer, 5′-CGCCATATGAGTCACTATATTGAATTAAC-3′; reverse primer 5′-CGCGGATCCGCCTAAGAGTTTGTTCAATTG-3′. Overexpression of the fusion protein was induced by adding 1 mM isopropyl-β-D-thiogalactoside at exponential phase and the incubation continued for 3 h at 37 °C. The induced cells were harvested by centrifugation (10,000 × *g*, 15 min, 4 °C), washed once with 50 mM Tris-HCl (pH 7.5), and subjected to sonication (3 × 1 min). The soluble fusion protein was purified to homogeneity by metal chelate chromatography on a Ni^{2+} column (3 ml) according to the manufacturer's instructions. The protein was eluted with 0.4 M imidazole in 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. Typically, 2–3 mg of homogenous Trx/100 ml of culture was obtained by this procedure. Both the histidine-tagged fusion protein and the recombinant Trx obtained after cleavage of the histidine tail by Factor Xa were indistinguishable in their spectroscopic properties and redox behavior.

Antiserum—Hyperimmune bovine anti-*H. pylori* antiserum was raised in cows immunized with a sonicate prepared from agar grown *H. pylori*. The IgG fraction was obtained by purification on Protein G (Amersham Pharmacia Biotech) using standard procedures. Anti-*H. pylori* thioredoxin antiserum was obtained from rabbits hyperimmunized with purified recombinant *H. pylori* thioredoxin using standard procedures. Anti-*E. coli* thioredoxin antiserum was obtained commercially (Sigma).

Reduction of Immunoglobulins and Mucin by the Trx System—Proteins (soluble porcine submaxillary gland mucin and immunoglobulins) to be reduced by the *H. pylori* Trx system were suspended in 50 mM Tris-HCl (pH 7.5) containing EDTA (1 mM). The reaction was performed at room temperature. After various periods of incubation the reaction mixture was subjected to alkylation by the addition of 4-fold molar excess iodoacetamide (in N_2-sparged 0.2 M Tris-HCl (pH 8.8)) over sulfhydryls to the sample. The mixture was left to incubate for 15 min under N_2 in the dark after which time an equal volume of double strength nonreducing sample buffer was added and the mixture boiled for 5 min prior to SDS-PAGE on 5–20% acrylamide gradient gels. The

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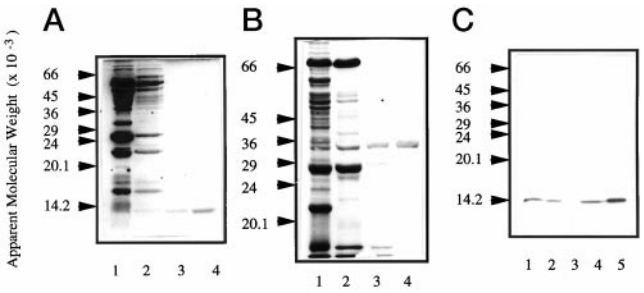

FIG. 1. **SDS-PAGE analysis of Trx and TR purification and subcellular distribution of Trx.** *Panel A* shows a Coomassie Blue-stained SDS-PAGE (15% acrylamide) electrophoretogram of samples from each stage of the purification of Trx; starting material (*lane 1*), DEAE-cellulose (*lane 2*), Sephadex G-50 (0.5 μg, *lane 3*), and Sephadex G-50 (1 μg, *lane 4*). *Panel B* shows an SDS-PAGE electrophoretogram stained with Coomassie Blue of samples from each step in the purification of TR; starting material (*lane 1*), DEAE column (*lane 2*), Cibacron blue (*lane 3*), and 2′,5′-ADP-agarose (*lane 4*). *Panel C* shows a Western blot of the subcellular localization of Trx in *H. pylori*; *lanes 1–5* represent whole *H. pylori*, cytoplasmic fraction, membrane fraction, PBS wash, and recombinant Trx, respectively, probed with anti-Trx antibodies affinity purified from hyperimmune bovine serum. The molecular weight marker proteins used were bovine serum albumin (M_r = 66,000), ovalbumin (M_r = 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r = 36,000), carbonic anhydrase (M_r = 29,000), trypsinogen (M_r = 24,000), and soybean trypsin inhibitor (M_r = 20,100), and lactalbumin (M_r = 14,200).

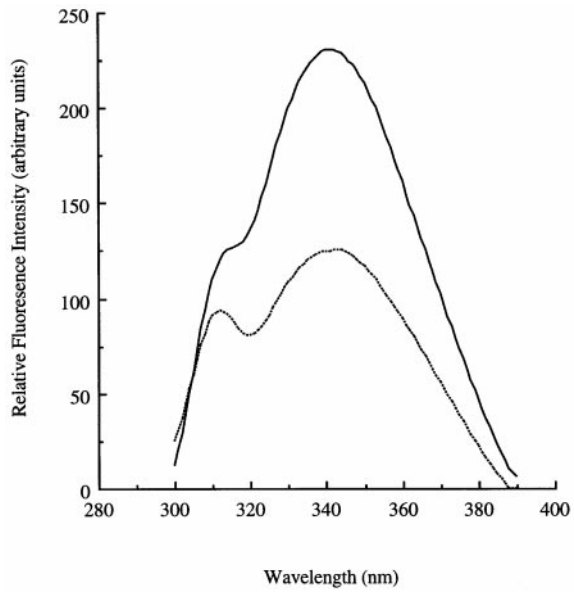

FIG. 2. **Fluorescence emission spectrum of reduced and oxidized forms of thioredoxin from *H. pylori*.** Thioredoxin (15 μg/ml) was reduced (—) or oxidized (- - - - -) by the addition of DTT (1 mM) or diamide (15 μM) to the sample, respectively, in 50 mM Tris-HCl (pH 7.3) containing EDTA (1 mM) for 10 min at 25 °C in a final volume of 2 ml prior to excitation of the samples at 280 nm using a light path of 1 cm. Fluorescence emission was recorded between 300 and 390 nm.

actual amounts and concentrations of the various components of the reaction are given in the figure legends where appropriate. Typically, the reaction was initiated by the addition of 3 μM immunoglobulin (Ig) to a mixture of recombinant *H. pylori* Trx (3-fold molar excess over Ig), TR (0.1 μM), and NADPH (400 μM). Samples of the reaction mixture (10 μl) were withdrawn at the times indicated in the figure legends and processed as described above. Gels were stained either with Coomassie Brilliant Blue or processed for Western blotting. Reduction of porcine submaxillary gland mucin was performed in a similar manner to that described for Igs and the reaction was monitored both spectrophotometrically (NADPH consumption) and by SDS-PAGE.

Subcellular Fractionation of H. pylori—Weakly cell-associated or soluble proteins were obtained by gently mixing a suspension of freshly harvested cells in PBS prior to centrifugation (10,000 × *g*, 10 min, 4 °C) to remove whole cells. The resulting supernatant was sterile filtered (0.2 μm Acrodisc, low protein binding) and stored at −70 °C. Membrane and cytosolic protein fractions were prepared essentially as described previously (9).

Measurement of Urease Activity—Urease activity was measured spectrophotometrically exactly as described previously (75).

Thioredoxin ELISA—Thioredoxin was detected in broth culture supernatants by coating ELISA plates (Nunc Maxisorp) with 50 μl of broth medium (concentrated 4-fold) overnight at 4 °C. Following washing (PBS) and blocking (3% BSA in PBS), bound thioredoxin was specifically detected by incubating the appropriate wells with polyclonal anti-*H. pylori* thioredoxin antiserum (1/1000) for 2 h at room temperature in a humidified atmosphere. After incubation with swine anti-rabbit IgG (1/1000; peroxidase conjugated) for 1 h at room temperature the bound conjugate was incubated with the substrate 3′,3′,5′,5′-tetramethylbenzidine according to the manufacturer's instructions (Sigma).

Sequence Analysis—Multiple sequence alignments were made with the Clustal program. Amino-terminal sequence analysis of purified *H. pylori* Trx and TR was performed by Aine Healy at the National Food Biotechnology Center, University College Cork, using an Applied Biosystems automated sequencer.

RESULTS

Purification, Subcellular Distribution, and Properties of Native Trx from H. pylori—Thioredoxin from *H. pylori* was purified to homogeneity in a two-step procedure which involved ion exchange on DEAE-cellulose followed by gel filtration over Sephadex G-50. An SDS-PAGE analysis of a sample taken from each step in the purification procedure is shown in Fig. 1 (*panel A*). Detection of Trx during its purification was based on its ability to reduce insulin disulfides, as described under "Experimental Procedures." Thioredoxin from *H. pylori* exists as a monomer with an apparent molecular mass of 12 kDa as determined by SDS-PAGE under reducing and nonreducing conditions. The NH_2-terminal 20 amino acid residues for Trx were found to be MSHYIELTEE*X*FESTIKKGV, where *X* represents an unidentified residue. A search of the protein data bases confirmed that we had purified Trx, entry HP 0824 in the *H. pylori* genome data base (12). Subcellular distribution studies demonstrated that Trx from *H. pylori* is largely a cytoplasmic protein (Fig. 1, *panel C*) with a small proportion of the total protein associated with the inner membrane fraction of the cell (not shown). Interestingly, Trx was detected in the soluble extracellular fraction which was obtained by briefly suspending the bacteria in PBS to remove loosely cell-associated material (Fig. 1, *panel C, lane 4*).

Overexpression of Trx in E. coli—Routinely, 2–3 mg of recombinant Trx was obtained per 100 ml of broth culture and the protein was expressed exclusively as a soluble cytoplasmic protein in *E. coli*. Removal of the His-tag from the purified material was achieved by digestion with Factor Xa (25 μg/mg protein) for 16 h at room temperature. To terminate the reaction the Factor Xa was removed from the mixture by passage through a small (1 ml) benzamidine-agarose column followed by purification of the recombinant protein (minus His-tag) on a Ni^{2+} column. Removal of the histidine tag resulted in a 2-kDa decrease in the molecular mass of the protein.

Spectral Properties—The fluorescence emission spectra of the native and recombinant Trxs were determined for both the reduced and oxidized forms of the protein. The spectral characteristics of the reduced forms of the native and recombinant protein (+His-tag) were identical as were those of their oxidized forms (not shown). The reduction of the recombinant *H. pylori* Trx by DTT (1 mM) resulted in a 1.8-fold increase in the tryptophan fluorescence intensity at 343 nm following excitation at 280 nm (Fig. 2). It has been demonstrated previously that thioredoxin from *E. coli* shows a larger increase in fluorescence intensity (3.5-fold) upon reduction of the active site cysteines due to the quenching effect of the active site disulfide

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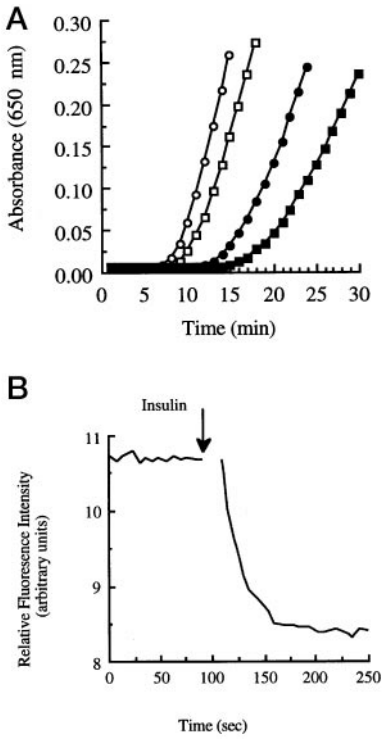

FIG. 3. **Reduction of insulin catalyzed by thioredoxin from *H. pylori* and *E. coli*.** *Panel A* shows the time course of insulin reduction in the presence of either Trx from *H. pylori* (○, 10 μM; □, 5 μM) or *E. coli* (●, 10 μM; ■, 5 μM). *Panel B* shows the fluorimeter tracing of reduced Trx oxidation by insulin. Using an excitation wavelength of 280 nm, the time course of fluorescence emission at 343 nm of *H. pylori* Trx (15 μg/ml) in 50 mM Tris-HCl (pH 7.3) containing EDTA (1 mM) was recorded in the absence and presence of added insulin. The *arrow* indicates the addition of insulin.

on the fluorescence of an adjacent tryptophan (13).

Reduction of Insulin—The ability of *H. pylori* Trx to catalyze the reduction of insulin by DTT was determined. Insulin reduction can be measured spectrophotometrically as an increase in turbidity due to precipitation of the free insulin B chain (88). We have compared the rates of porcine insulin reduction by DTT in the presence of *H. pylori* and *E. coli* Trxs. Both Trxs show activity as disulfide reductases with insulin as substrate (Fig. 3, *panel A*). Interestingly, Trx from *H. pylori* was approximately four times as efficient at reducing insulin than equivalent amounts of *E. coli* Trx when incubated with DTT (Fig. 3). In addition, the initial rate of insulin reduction was greater with *H. pylori* Trx compared with Trx from *E. coli*. These observed redox activities (Fig. 3) and the differential spectral properties of reduced and oxidized Trx (Fig. 2) enabled us to examine the oxidation of fully reduced Trx by insulin and to assay the process continuously using a fluorimeter. Fluorescence spectroscopy of reduced thioredoxin re-oxidation by insulin demonstrated that the process occurred rapidly upon addition of an equal amount of insulin (Fig. 3, *panel B*) and that the thioredoxin was oxidized within 2 min of the initiation of the reaction. Taken together, these data indicate that we had a functional recombinant molecule which behaved in a manner identical to the purified native Trx.

Purification and Properties of TR from H. pylori—Thioredoxin reductase was purified to homogeneity using a three-step purification procedure involving ion-exchange chromatography over DEAE-cellulose, Cibacron blue 3GA, and affinity purification over 2′-5′-ADP-agarose. During purification the activity of TR was monitored by its ability to catalyze NADPH-dependent reduction of the disulfide bond in 5,5′-dithiobis(2-nitrobenzoic acid) as described previously (11). Thioredoxin reductase activity eluted as a distinct sharp peak from DEAE-cellulose upon the application of a salt gradient. Active fractions were pooled, dialyzed, and applied to a small column of Cibacron blue 3GA and eluted in a highly purified form with KCl. Finally, the enzyme was purified to homogeneity by affinity chromatography over 2′,5′-ADP-agarose. An SDS-PAGE electrophoretic profile of each step in the purification procedure is shown in Fig. 1 (*panel B*). A native molecular mass of approximately 70,000 Da was determined for TR on a calibrated column of Sephacryl S-300 (results not shown). The apparent molecular mass determined by analytical reducing SDS-PAGE of the purified activity was 35 kDa (Fig. 1, *panel B, lane 4*) thus indicating that the *H. pylori* enzyme consists of two identical or similar subunits held together by a disulfide bond. NH_2-terminal amino acid analysis of this peptide yielded the sequence MID*X*AIIGGGPAGL*X*AGLYA, where *X* represents an unidentified residue. This sequence corresponds to entry HP 825 in the *H. pylori* genome data base. The pure enzyme (TR) obtained after affinity chromatography on 2′,5′-ADP-agarose showed absorption maxima at 290, 350, and 420 nm (not shown). Thioredoxin reductase from *H. pylori* was strictly dependent on NADPH and was inactive in the presence of NADH (not shown), a feature common to most thioredoxin reductases described to date. In addition, the thioredoxin reductase reaction ($Trx\text{-}S_2$ + NADPH + $H^+ \leftrightarrow Trx\text{-}(SH)_2$ + $NADP^+$) was reversible by addition of excess $NADP^+$ (not shown).

Comparisons with Other Thioredoxins and Thioredoxin Reductases—The alignment of the deduced amino acid sequences encoded by the *trxB* gene from *H. pylori* and other selected species is shown in Fig. 4*A*. Greatest homology (>51%) between *H. pylori* Trx and Trx from other species is seen with *Bacillus subtilis, Saccharomyces aureus*, *Anabaena* sp., and the red alga *Cyanidium caldarium*. The redox-active site CGPC of Trx is highly conserved. Interestingly, Trx shares good homology to Trx from *B. subtilis*, recently shown to be an essential protein for the organism which is induced by multiple stresses (87). On the other hand *H. pylori* TR is most homologous (44%) to TR from *Clostridium litorale* (not shown). In addition, the *H. pylori* enzyme possesses 2 conserved motifs responsible for binding of FAD near the NH_2 terminus (G*X*G*XX*G) and NADPH near the middle of the protein (GGGD*X*A) as well as a redox active center (CATC). There is a fourth conserved domain with no homology to known motifs, located at the COOH terminus of the protein (G*X*FAAGD) (not shown).

Reduction of Human Polyclonal IgG by the Trx System of H. pylori—In order to study the patterns of reduction of human Igs, samples were reduced and alkylated over various periods of time and analyzed by both SDS-PAGE and Western blotting. Fig. 5 (*panel A*) shows the results obtained when human polyclonal IgG was reduced by the Trx system of *H. pylori*. Overall the SDS-PAGE analysis demonstrated that IgG was reduced almost to completion with time. In addition to the intact immunoglobulin (H_2L_2, M_r: 146,000–170,000) and the major fragments of heavy (H, M_r: 51,000–60,000) and light (L, M_r: 22,000–25,000) chains, the small number of minor bands, which appear transiently, presumably represent the formation of mixed disulfides (*e.g.* H_2L, M_r: 124,000–145,000; H_2, M_r: 102,000–120,000; HL, 73,000–85,000) and these are identified in the margins of the figures, where appropriate. It is clear that human IgG is an efficient substrate for this system. IgG was completely reduced after a 3-h incubation (at room temperature) using equimolar amounts of Trx and Ig in the presence of TR and NADPH. Similarly, an identical cleavage pattern of IgG was observed when the reaction was monitored by Western blotting and probed and developed with anti-human IgG and

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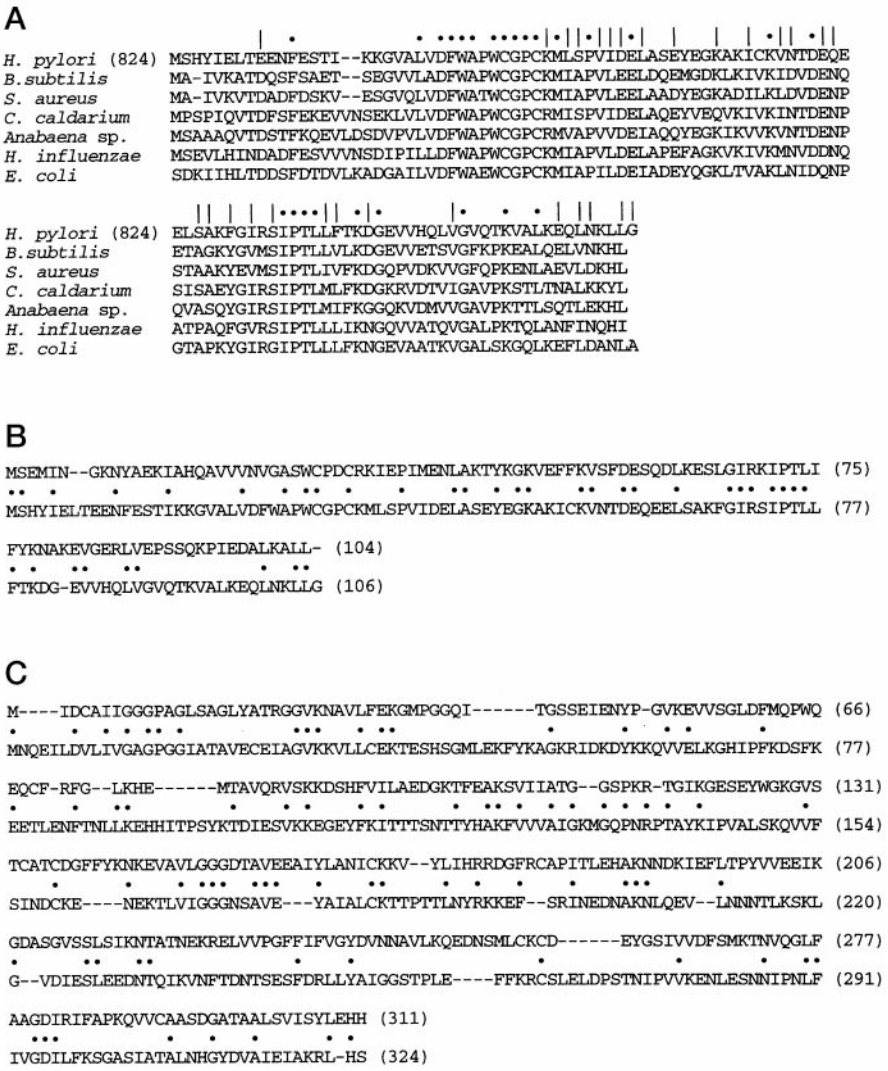

FIG. 4. **Amino acid sequence alignment of thioredoxin and thioredoxin reductase.** Multiple alignment of Trx (HP 824) from *H. pylori* and other species is shown in *panel A*. *Panel B* shows the paired alignment of the two species of Trx from *H. pylori* (HP 824, *lower*; HP 1458, *upper*). *Panel C* shows the homology between the two species of TR from *H. pylori* (HP 825, *top*; HP 1164, *lower*). The *vertical bars* indicate conservative substitutions/regions of strong similarity as opposed to identity and the *dots* indicate identical residues.

ECL, respectively (not shown). The reduction of Ig was dependent on reduced thioredoxin as neither oxidized thioredoxin nor thioredoxin reductase alone was able to reduce IgG (not shown). Furthermore, analysis of the subclass specificity of the reduction process revealed that thioredoxin could efficiently reduce IgG_1, IgG_2, IgG_3, and IgG_4 as shown by Western blotting with antisera raised against purified IgG subclasses (Fig. 5, *panels B-E*). The Western blotting data demonstrated that the interchain disulfide bonds of different subclasses of IgG were reduced in a time-dependent manner by the Trx system but with significant kinetic differences. IgG_1 was reduced rapidly to H and L chains (by 10–20 min). Transient appearance of mixed disulfides (H_2L, H_2, and HL) was seen also and these are indicated in Fig. 5 (*panel B*). Clearly the 4 interchain disulfides of IgG_1 are readily reduced, whereas the remaining subclasses were less susceptible to reduction in the following preferential order; $IgG_3 > IgG_4 > IgG_2$. Almost complete reduction of IgG_3 (Fig. 5*D*) and IgG4 (Fig. 5*E*) was seen by 40–60 and 60–90 min, respectively. IgG_2 (Fig. 5*C*) was the subclass most resistant to reduction although reduced H chains were apparent after only 5 min of incubation. In addition, fewer intermediate mixed disulfides were seen with IgG_2 as substrate compared with the other subclasses. The kinetic differences observed in cleavage patterns are most likely due to structural differences in the Igs where steric constraints will effect both the accessibility and interaction of Trx with sulfhydryls in addition to altering the susceptibility of these thiols to reduction. Finally, it appears from our data that *H. pylori* Trx is unable to reduce the intrachain disulfide bonds of the heavy (H) and light (L) chains. Only when the alkylated Trx-reduced H and L chains were subjected to complete reduction by 2-mercaptoethanol or DTT were the fully reduced H and L chains observed (data not shown). Cleland (14) has shown that DTT, because of its low redox potential, is capable of maintaining monothiols completely in the reduced state and of reducing disulfides quantitatively.

Reduction of IgA—The reduction of IgA_1 and the secretory component (SC) of sIgA was performed and monitored exactly as described for IgG. The cleavage patterns of IgA_1 and the secretory component are shown in Fig. 6. Monomeric nonsecretory IgA_1 was readily reduced by the Trx system as can be seen from the appearance of the expected cleavage fragments with time (Fig. 6, *panel A*). The anti-IgA antibody only weakly recognized the L chain of IgA and this was only seen upon overexposure of the blot (not shown). The Trx-mediated reduction of dimeric sIgA proceeded more slowly than reduction of IgG, monomeric non-secretory IgA or polymeric IgA (not shown) presumably due to steric hindrance imparted by the SC and J-chain. Similarly, reduction of the SC of dimeric sIgA was observed using anti-SC antiserum (Fig. 6, *panel B*). The fragment appearing at 183 kDa most likely represents SC bound to monomeric sIgA (H_2L_2) as opposed to SC bound to the dimeric sIgA ($[H_2L_2]_2$) as indicated in the figure. Free SC (68 kDa) is released from 5 min of incubation. From our data it appears

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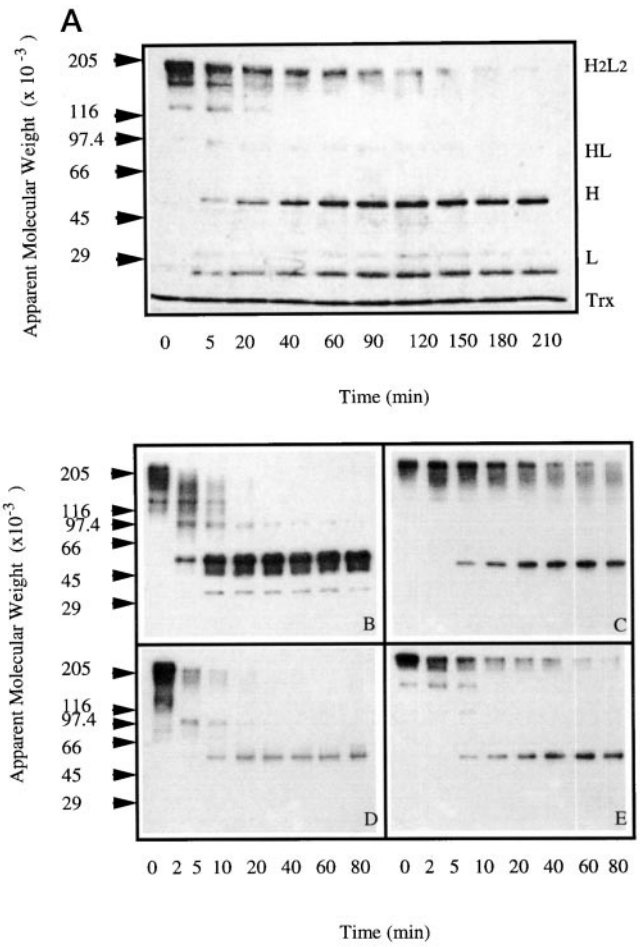

FIG. 5. **Reduction of polyclonal human IgG by the Trx system of *H. pylori*.** *Panel A* shows a time course of the reduction of human polyclonal IgG by the Trx system. After boiling for 5 min 20 μl of each sample was loaded into the wells of a gradient (4–20%) SDS-polyacrylamide gel. Proteins were detected in the gel by staining with Coomassie Brilliant Blue. The reduction of the four subclasses of human IgG (IgG_1, IgG_2, IgG_3, and IgG_4) was examined by Western blotting and the results are shown in *panels B-E*, respectively. The molecular weight markers used were the same as used in Fig. 1, with the exception of myosin (M_r = 205,000), β-galactosidase (M_r = 116,000), and phosphorylase *b* (M_r = 97,400).

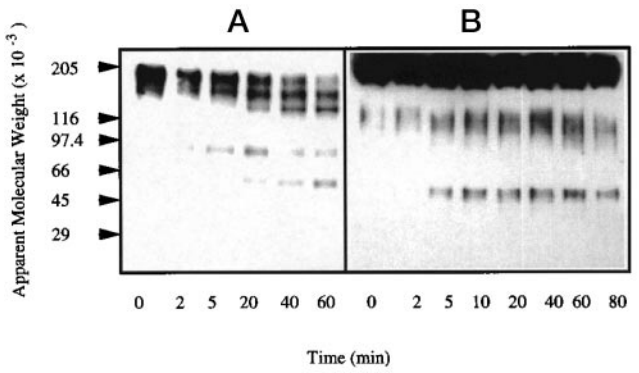

FIG. 6. **Reduction of human IgA by the Trx system of *H. pylori*.** *Panels A* and *B* show Western blots of the time course of Trx-mediated reduction of human IgA_1 and human sIgA, respectively. Reduction of IgA_1 and secretory component were monitored by developing the blots with peroxidase-conjugated anti-IgA (α-chains) and anti-secretory component antibody, respectively. The molecular weight markers used were identical to those described in the legend to Fig. 5.

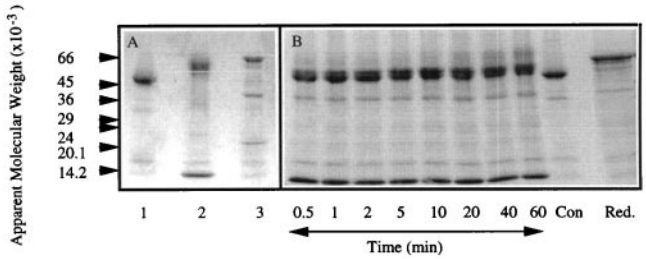

FIG. 7. **Reduction of mucin by the Trx system of *H. pylori*.** Reduction of porcine submaxillary gland mucin by the Trx system of *H. pylori* was examined by SDS-PAGE. *Panel A* shows the results obtained after a 2-h incubation of mucin with the Trx system (*lane 1*, untreated mucin; *lane 2*, Trx-treated mucin; *lane 3*, 2-mercaptoethanol-treated mucin). *Panel B* shows the time course of mucin reduction by Trx (0.5–60 min). Also shown is the untreated starting material after the 60-min incubation (*Con.*) and the fully reduced protein (*Red.*). The molecular weight markers used are described in the legend to Fig. 1.

that removal of the secretory component may represent a rate-limiting step in the reduction of sIgA, as a considerable amount of the molecule remained bound to the dimeric sIgA even after 80 min of incubation. Presumably, however, the rate of reduction would be much greater if the reaction was performed at 37 °C rather than room temperature (21 °C) as described.

Reduction of Mucin—Upon reduction of soluble porcine submaxillary gland mucin by the Trx system the apparent molecular weight of the mucin was increased as demonstrated by analytical SDS-PAGE (Fig. 7*A*). This observed anomalous increase in the molecular mass of mucin upon Trx-mediated reduction (Fig. 7*A, lane 2*) is interpreted as the presence of residual intact intrachain disulfides which give the partially linearized mucin an electrophoretic mobility less than that of the nonreduced material (Fig. 7*A, lane 1*). Similarly, upon complete reduction of the mucin by 2-mercaptoethanol the electrophoretic mobility of the material is decreased yet further due to complete linearization of the mucin structure (Fig. 7*A, lane 3*). Fig. 7*B* shows the time course of mucin reduction by the Trx system and the associated gradual decrease in electrophoretic mobility. Similar anomalous electrophoretic mobilities have been found for the partially and fully reduced forms of IgG H and L chains. This behavior is thought to be due to the loss of domain compactness induced by reduction (15, 39).

Two-dimensional SDS-PAGE Analysis of Thioredoxin Expression by H. pylori—Broth cultures *of H. pylori* were incubated in the absence or presence of bovine anti-*H. pylori* IgG for various periods of time. Fig. 8*A* shows a two-dimensional SDS-PAGE analysis/Western blot analysis of the time course of expression of intracellular thioredoxin before, and for various periods of time after the addition of antibody. Interestingly, the expression of intracellular thioredoxin exhibits a biphasic response with time with the basal level rapidly decreasing 15 min after the addition of antibody followed by a transient increase observed at 30 min. After this time intracellular thioredoxin decreased to almost undetectable levels by 2–4 h of incubation with antibody. Subsequent analysis of the broth culture supernatant for the presence of thioredoxin using an ELISA developed with anti-*H. pylori* thioredoxin IgG revealed that the apparent disappearance of intracellular thioredoxin was in fact due to secretion of the protein from the cell in response to incubation with anti-*H. pylori* IgG. It is clear from Fig. 8*B* that thioredoxin accumulated in the extracellular medium with time after exposure of the cells to anti-*H. pylori* antiserum. Importantly, it appears that the accumulation of thioredoxin in the medium is due to specific secretion rather than lysis of the bacteria as the extracellular levels of urease activity remained essentially constant throughout the duration of the experiment (Fig. 8*B*). In support of this view, Western blotting, developed with hyperimmune anti-*H. pylori* antiserum, demonstrated

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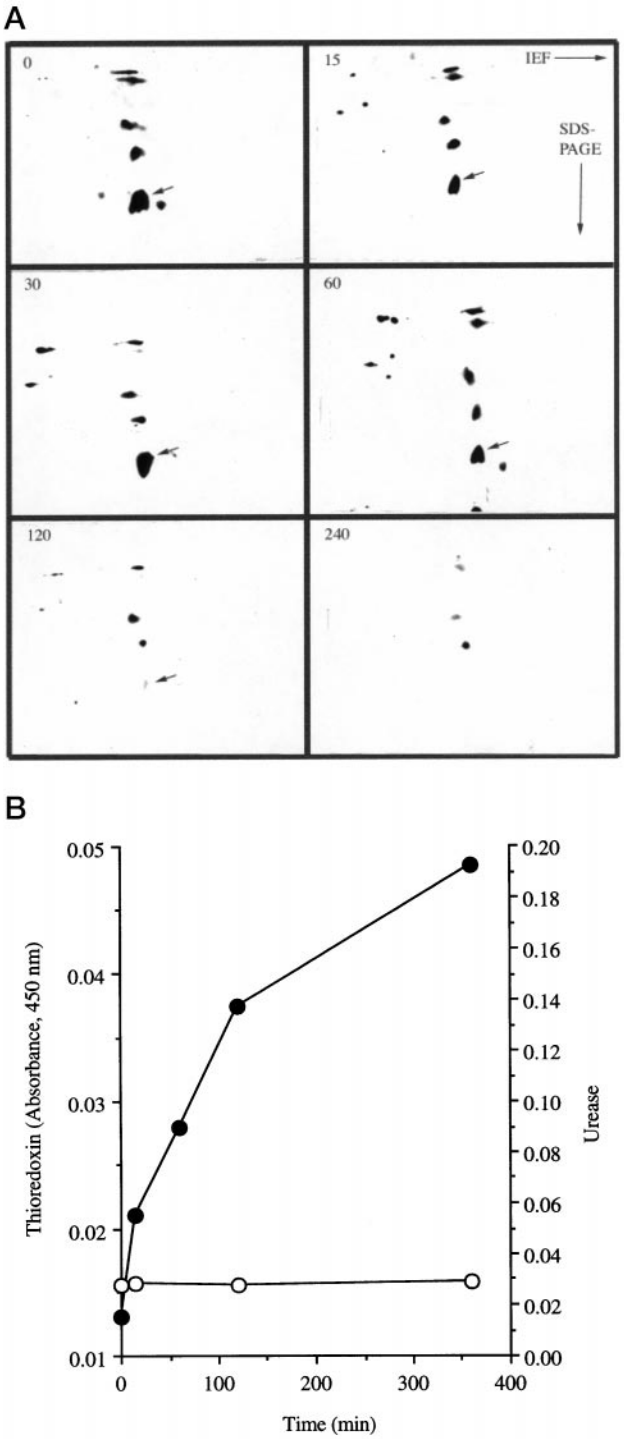

FIG. 8. **Thioredoxin expression in response to stress.** *Panel A* shows the time course of thioredoxin expression in broth grown *H. pylori* in response to exogenously added bovine anti-*H. pylori* IgG (0.1 mg/ml). The suspension of bacteria was incubated at 37 °C and at various periods of time after the addition of antibody 0.5-ml samples of the suspension were removed and immediately centrifuged (10,000 × *g*, 4 min) to pellet the bacteria. After removal of the supernatant the pellet of cells was solubilized in IEF sample buffer and subjected to two-dimensional SDS-PAGE followed by Western blotting. Thioredoxin was detected by incubating the blots with polyclonal anti-*E. coli* thioredoxin IgG which cross-reacts with *H. pylori* thioredoxin. The position of thioredoxin is indicated by the *arrow*. The *numbers* in each frame indicate the time (min) at which samples were removed from the incubation with antibody. *Panel B* shows the time course of the accumulation of thioredoxin (*filled symbols*) and urease (*open symbols*) in the broth culture supernatant in response to the addition of polyclonal anti-*H. pylori* IgG to broth grown *H. pylori*. The presence of thioredoxin in the broth culture medium was detected by ELISA as described under "Experimental Procedures."

that there was no apparent time-dependent accumulation of multiple *H. pylori* antigens in the broth culture supernatant over the duration of the experiment (not shown). Furthermore, the viability of the bacteria was unaffected by the presence of anti-*H. pylori* IgG as assessed by subculture of the organism after completion of the experiment. Moreover, a control experiment demonstrated that urease activity was unaffected by the polyclonal anti-*H. pylori* IgG used, therefore the detectable urease activity in the broth supernatant represented active urease enzyme rather than residual activity as a consequence of antibody-mediated inhibition. In addition, low levels of thioredoxin reductase activity (0.3 ± 0.08 nmol/NADPH oxidized/min/50 μl of broth supernatant) were detected in the broth culture supernatant. Unlike thioredoxin, however, extracellular levels of thioredoxin reductase remained constant and did not increase with time upon exposure of the bacteria to antibody.

DISCUSSION

This paper describes the isolation and characterization of electrophoretically pure thioredoxin (HP 824) and thioredoxin reductase (HP 825) from the gastric pathogen *H. pylori* and the cloning and overexpression of thioredoxin in *E. coli*. This is the first reported characterization of a functional thioredoxin system in a gastric pathogen. The results show that the Trx system can specifically reduce interchain disulfides in insulin, mucin, IgG, and IgA. Of particular note is the finding that, in response to a variety of applied extracellular stresses, the expression of intracellular Trx is dramatically altered by *H. pylori*, an observation which suggests that Trx behaves as a stress response element in this organism. Importantly, the accumulation of Trx in the medium of broth grown bacteria in response to physical, chemical, or biological insults suggests that this molecule has the ability to protect the bacterium by initiating the process of catalytic reduction of susceptible target proteins. This suggestion is supported by the detection of measurable amounts of thioredoxin reductase activity also in broth culture supernatants thus equipping the bacterium with a functional extracellular thioredoxin system.

Interestingly, while there was an increase in the amount of thioredoxin secreted in response to stress there was no detectable increase in the extracellular levels of TR over the time course of the experiments. The significance of the quantitative changes in secretion observed for Trx but not for TR remain to be established, however, the induction and secretion of Trx alone would increase the capacity of the bacterium to react to stress. Differential expression of Trx, TR, and other redox active proteins in response to various stressors has been observed in eukaryotic and mammalian cells (81–84) and, paradoxically, oxidative stress has been shown to result in the down-regulation of TR activity but to increase Trx activity in epithelial cells (85). Differential expression of Trx and TR by *H. pylori* may occur. Clearly a portion of such an adaptive response would be regulated at the transcriptional level, however, the transcription factor(s) that regulates the expression of Trx/TR in *H. pylori* have yet to be identified. Alternatively, induction of Trx synthesis under conditions of stress may result in transient feedback inhibition of a putative redox-sensitive transcription factor which, subsequently, may be reactivated by a transient imbalance in the redox status of the cell in a manner analogous to the regulation of the OxyR transcription factor in *E. coli* (86). Such an imbalance would likely occur upon secretion of intracellular Trx in response to stress inducing stimuli. Yet another possibility is that Trx and TR from *H. pylori* are under the control of different promoters and as such these promoters may show a differential response to various stimuli as has been documented for Trx expression in *B. sub-*

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tilis (87). Clearly, identification of such factors would aid our understanding of redox control mechanisms in *H. pylori*.

Thioredoxin-mediated catalytic reduction of host immunoglobulins would clearly facilitate immune evasion. This may very well account for the observation that there is little or no host antibody deposition on *H. pylori in vivo* (*e.g.* Ref. 32). Moreover, it is well documented that even partial chemical reduction of human and rat immunoglobulin results in loss of its biological activity (33–35). The basis of many of the effects of mild chemical reduction on the various effector functions of IgG are explained largely by the reduction of the single disulfide bond between heavy chains in the hinge region of IgG resulting in destabilization of the CH2 region of the molecule and consequent modification of the quaternary structure. For example, initiation of complement activation requires the presence of the inter-H chain disulfide bonds in the hinge region (36). Chemical reduction has been shown also to be inimical to the interaction between IgG and various cells, including monocytes (37), neutrophils (38), B-cells (49), and macrophages (50). Similarly, reduction of IgA would be expected to compromise its biological activity. The importance of IgA in mucosal secretions is well established and recognized generally to play a key role as an initial barrier to infection. Reduction of proteins imparts enhanced susceptibility to proteolysis due to loss of structure and consequent unfolding. In this regard, reduction and removal of the heavily glycosylated, cysteine-rich protective SC from sIgA, which increases the resistance of sIgA to proteolysis (51), would undermine its crucial function in external secretions.

With respect to *H. pylori,* however, the effector functions of IgA appear to be less well defined. Contrasting data from several groups have demonstrated the presence of widely varying IgA/sIgA titers in different populations of *H. pylori* infected subjects (*e.g.* Refs. 76 and 78). Indeed, it has been demonstrated that antibody-independent mechanisms of immunity can protect against *Helicobacter* infection (77, 79), whereas others hold the view that specific mucosal antibodies may prevent overgrowth of *H. pylori* thereby reducing the risk of gastric malignancies (80), observations which suggest that there is a tenuous relationship between specific mucosal antibody and protection from infection. The experimental approach adopted in many of these studies (ELISA) to determine antibody levels, however, would not discriminate between active and inactive antibody species. Therefore, it is not inconceivable that the fraction of mucosal antibody which comes into contact with *H. pylori* is partially or completely inactivated via complete or incomplete catalytic reduction and that this fraction represents only a small percentage of the total antibody present in the gastric lumen. We propose that gross modification to antibody structure would be an unlikely occurrence. Rather, catalytic reduction would only conceivably occur in the immediate microenvironment of the bacterium where it is required. Also, the thioredoxin system could only remain functional so long as the respective components of the system were in close proximity to one another.

H. pylori has been shown by several laboratories to adversely affect the physical and chemical properties of gastric mucins (26–29, 57, 58) and that eradication of the bacterium results in restoration of the viscoelastic and hydrophobic protective properties of mucus (30, 31, 56). Although still a controversial and disputed issue (see Refs. 59–61) the weight of experimental evidence greatly favors *H. pylori*-mediated alterations to mucin structure/function. Moreover, it has been well documented that *H. pylori* inhabits the adherent (water insoluble) mucous gel layer in addition to the more soluble viscous mucin of the lumen. So how does *H. pylori* gain access to the adherent mucus gel, a substance known to be impermeable to proteins with a molecular size greater than 17,000 Da (67)? As the motility of *H. pylori* is affected by the viscosity of the medium it inhabits (25) focal disruption of the mucus barrier would clearly facilitate rapid passage of the bacterium. Studies *in vitro* suggest that loss of gel structure may arise due to *H. pylori*-mediated proteolytic or phospholipase activity, alterations in local pH (62, 63), or reversible modifications to patterns of gastric mucin glycosylation (64). Although this contentious issue has yet to be resolved unequivocally, we propose an alternative mechanism. Conceivably, based on the data presented in this paper, *H. pylori* could gain access to the impenetrable adherent mucous layer by inducing focal disruption to the barrier by catalytically reducing the disulfide bonds present in the cysteine-rich regions responsible for cross-linking mucin monomers (19, 65, 66). These cysteine-rich domains are susceptible to both proteolytic attack and thiol agents (67). In addition, thioredoxin would represent an ideal candidate molecule to fulfil this task given its small size (12 kDa) and reducing capacity. Clearly, reduction of mucin disulfides would thus facilitate the process of colonization as a direct consequence of the loss of gel-forming properties of polymeric mucin which provide protection for the exposed delicate surfaces of the gastric epithelia from microbial and physical insults (18–24).

Clearly, any reductive redox activity mediated by Trx in the vicinity of antibodies and/or mucin could result in complete or partial catalytic reduction of the disulfides present in these molecules. It is clear that human Igs and mucin are reduced readily by the Trx system of *H. pylori*. Interestingly, all four subclasses of IgG are reduced by the Trx system of *H. pylori* in contrast to the reported inability of the Trx system from *E. coli* to reduce human IgG_2 (39). Of course, if such a reductive process were to occur *in vivo* it would require that the Trx system be capable of exerting its effects extracellularly. We have shown that Trx is present in the PBS-soluble extracellular (water soluble) fraction from freshly harvested *H. pylori* in addition to being secreted in an apparently specific manner when the bacteria are subjected to a variety of stresses and therefore reasonably speculate that Trx may be secreted from the cell or remain surface-associated, as required. The mechanism of Trx secretion from the bacterium is not clear at present. Although Trx lacks a typical signal sequence there are a number of ways in which it could be released from the cell. Principal among these are specific secretion pathways (52, 73), a potential type IV secretory mechanism (74), spontaneous autolysis (53, 71), and the shedding of membrane vesicles (68–70, 73) some of which have been shown to account for the extracellular localization of several cytoplasmic *H. pylori* proteins (*e.g.* Refs. 68 and 70). These *in vitro* observations of potential mechanisms for shedding/releasing *H. pylori* antigens are given credence by the localization *in vivo* of various *H. pylori* proteins, such as urease and HspB (53, 72) in the lamina propia of infected individuals. Moreover, it has been suggested that the extracellular release of *H. pylori* antigens may serve as a means of evading the host immune response and, with the presence of an extracellular functional thioredoxin system, a mechanism to incapacitate host antibody.

It is likely that the Trx system of *H. pylori* has an important function in this bacterium particularly in view of the fact that it possesses limited means for manipulating and maintaining a reducing intracellular environment with the exception of superoxide dismutase and catalase. *H. pylori* is a microaerophile and lives in an environment of low oxygen tension. Such an environment encourages optimal conditions for reductive reactions although it is likely that the bacterium has the ability to adapt to conditions of variable oxygen tension (16). Unlike many other prokaryotes, *H. pylori* does not appear to possess

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the enzymes to generate glutathione nor does it possess other thiol reductants such as glutaredoxin. Accumulating evidence indicates that Trxs from several species have multifunctional roles, however, the precise functions of Trxs have yet to be established unequivocally. One difficulty in assigning specific *in vivo* functions to Trx will be compounded by its many proposed regulatory functions (41–48) and the wide variety of substrates with which it interacts (*e.g.* Ref. 40). Despite the similarities of the conserved C*XX*C motif among Trxs from different species, the various members differ strongly in their redox potentials (−122 to −270 mV). Determination of the redox potential of *H. pylori* Trx will give an indication of the redox capacity of the molecule and enable us to investigate further its functions *in vivo*.

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