Glutaredoxins are ubiquitous proteins that catalyze the reduction of disulfides via reduced glutathione (GSH). *Escherichia coli* has three glutaredoxins (Grx1, Grx2, and Grx3), all containing the classic dithiol active site CXXC. We report the cloning, expression, and characterization of a novel monothiol *E. coli* glutaredoxin, which we name glutaredoxin 4 (Grx4). The protein consists of 115 amino acids (12.7 kDa), has a monothiol (CGFS) potential active site and shows high sequence homology to the other monothiol glutaredoxins and especially to yeast Grx5. Experiments with gene knock-out techniques showed that the reading frame encoding Grx4 was essential. Grx4 was inactive as a GSH-disulfide oxidoreductase in a standard glutaredoxin assay with GSH and hydroxyethyl disulfide in a complete system with NADPH and glutathione reductase. An engineered CGFC active site mutant did not gain activity either. Grx4 in reduced form contained three thiols, and treatment with oxidized GSH resulted in glutathionylation and formation of a disulfide. Remarkably, this disulfide of Grx4 was a direct substrate for NADPH and *E. coli* thioredoxin reductase, whereas the mixed disulfide was reduced by Grx1. Reduced Grx4 showed the potential to transfer electrons to oxidized *E. coli* Grx1 and Grx3. Grx4 is highly abundant (750–2000 ng/mg of total soluble protein), as determined by a specific enzyme-link immunosorbent assay, and most likely regulated by guanosine 3′,5′-tetraphosphate upon entry to stationary phase. Grx4 was highly elevated upon iron depletion, suggesting an iron-related function for the protein.
A null mutant for the three yeast monothiol glutaredoxins was not viable (15). Therefore, these enzymes must have very specific targets, which are not substrates for the remaining two yeast dithiol glutaredoxins (15). The monothiol Grx5 is involved in the formation of iron-sulfur clusters (16), whereas its absence prevents respiratory growth and causes accumulation of free iron in the cell and constitutive oxidation of proteins (16). ydhD, an open reading frame with 37% amino acid sequence identity to yeast Grx5, exists in the genome of *E. coli*. To elucidate the function of ydhD, we cloned the gene, overexpressed and purified the respective protein, studied its reactivity with other redox factors, and estimated its protein levels by sandwich ELISA. ydhD has recently been suggested as essential for aerobic growth in rich media (17). In the accompanying paper (18) the solution structure of Grx4 has been determined.

**Materials and Methods**

**Chemicals**—Streptavidin-alkaline phosphatase-PQ was from Chemicon International. p-nitrophenyl phosphate was from Sigma. All other chemicals were purchased from commercial sources. *E. coli* TfrX (PAPS reductase, 11, 12), Grx1 (20), Grx1C14S (6), Grx2 (7), and Grx3 (10) were from homogeneous preparations from our laboratory.

**Strains and Media**—Strains were grown on LB plates supplemented whenever needed with ampicillin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (20 μg/ml), or chloramphenicol (20 μg/ml). The bacterial strains of this work are listed in Table I. For most experiments examined expression levels of Grx4 by ELISA, apart from those of Table III and Fig. 4A, cells were grown first in 10 ml of LB medium, harvested, and centrifuged (12,500 g, 20,000 × *g* for 30 min) at 4 °C. Ammonium sulfate supernatants were dialyzed overnight against 20 mM Tris-HCl, pH 8.0, 1 mM EDTA and chromatographed on DEAE-Sepharose equilibrated with the same buffer. The column was washed with 50 mM Tris-Cl, pH 8.0, 1 mM EDTA, and bound Grx4 was eluted with a gradient of NaCl (0–500 mM). Fractions containing Grx4 (as determined by SDS-PAGE) were concentrated with YM Diaflo ultra filters (cut-off of 3 kDa), incubated for 45 min with 5 mM DTT at room temperature.
temperature, and chromatographed on a Sephadex G-50 superfine gel filtration column in 200 mM potassium phosphate, 50 mM Tris-Cl, pH 8.0, 1 mM EDTA.

**Circular Dichroism Spectroscopy**—CD experiments were performed using an AVIV Associates model 62DS circular dichroism spectrometer. Measurements were performed with a 0.1-cm path length cuvette in 5 mM potassium phosphate, 100 mM KCl, pH 7.4. Protein concentrations were 21 and 17 μM for Grx1 and Grx4, respectively. The mean residue ellipticity [θ] was expressed as units of 10^4deg cm^2 dmol^-1. Wave-length spectra were recorded from 195 to 260 nm at 25 °C. Data were collected every nanometer, with an averaging time of 2 s and a bandwidth of 1.5 nm averaging over six repeated scans. Thermal denaturations were monitored by measuring the change in ellipticity at 222 nm with increasing temperature from 5 to 90 °C at a speed of 3 °C/h. Data were smoothed and degree each, with an autocorrelation time and a 1.5-nm bandwidth. To estimate thermodynamic parameters, models derived from the van’Hoff equation and the standard equation for Gibbs free energy (24) were fitted directly to the experimental data using SigmaPlot (SPSS Inc.).

**Construction of Grx4 Active Site Mutants—** grxD from the pGEM-T vector was amplified using primers Grx4 F-NcoI and Grx4 SGSF RC (5’-CAA TGG GTG TGC TGG-3’) or Grx4 RC-BamHI and Grx4 SGSF S (5’-CAG CTT TCT CTC CCA GCC-3’). For both reactions, PCR conditions were 95 °C for 4 min, then 30 cycles of 95 °C for 30 s, 42 °C for 30 s, and finally 72 °C for 10 min. The two resulting PCR fragments were used together as templates for Grx4 F-NcoI and Grx4 RC-BamHI (95 °C for 4 min, then 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and finally 72 °C for 10 min) to give a Grx4 product encompassing the Grx4 SGSF, which could be subcloned to pET15b after digestion with NcoI and BamHI. The other two mutants were constructed in exactly the same manner as primer couples for the active site mutations Grx4 CPYC F (5’-CCG AGC TGC TGC CGG-3’) and Grx4 CPYC RC (5’-ACT GCC TGG GCA CAA CGG TGC-3’). Preparation of Differently Oxidized Grx4 Species—Recombinant Grx4 obtained after purification was fully reduced by treatment with 5 mM DTT for 1 h at 37 °C, in 200 mM potassium chloride, 50 mM Tris-Cl, pH 8.0, 1 mM EDTA. Reduced Grx4 was oxidized after addition of 2 mM GSSG, 45 min at room temperature in the same buffer. The GSSG-oxidized Grx4 was desalted in 1 mM EDTA, 50 mM Tris-Cl, pH 8.0, and served as a substrate for TrxR in a mixture composed of 100 mM TrxR, 0.2 mM NADPH, 1 mM EDTA, and 0.1 mg/ml bovine serum albumin (BSA) in 50 mM phosphate buffer, pH 7.0 (final assay volume of 500 μl). The decrease at 410 nm was monitored as evidence of reduction by Grx4 by affinity chromatography and quantification of Grx4 by sandwich ELISA was carried out by methods previously described for E. coli Grx2 (25). The effect of cell-free extracts on the recovery of Grx4 was examined. Known concentrations of standards were diluted in cell-free extracts, and the measured concentrations were compared with those expected (26). Parallelism tests between sample and standard curves were performed for all samples (26). Intra-assay variations were determined by replicates of the same sample in the same plate and inter-assay variation by the same sample analyzed at different days (27).

**RESULTS**

**Grx4 Sequence Analysis and Comparison**—Analysis of the E. coli genome revealed the existence of ydhD, an open reading frame encoding a protein with significant amino acid sequence homology to known monothiol glutaredoxins (Fig. 1). Due to its high homology to yeast monothiol glutaredoxins, we renamed the gene grxD and the putative protein Grx4. The protein displays a 37% sequence identity to the yeast monothiol Grx5 but is less similar to the classic dithiol Grx1 and Grx3 from...
second model gave an improved overall fit of the thermal denaturation data for both proteins with almost identical $T_m$ values and enthalpies (Fig. 2B). For Grx4, the fit provided by the second model gave $T_m = 66.7 \pm 0.2 \, ^\circ\text{C}$ and $\Delta H_m = -54.2 \pm 1.5 \, \text{kcal}\cdot\text{mol}^{-1}$, whereas for Grx1, $T_m = 67.7 \pm 0.1 \, ^\circ\text{C}$ and $\Delta H_m = -58.4 \pm 1.2 \, \text{kcal}\cdot\text{mol}^{-1}$. The estimated values of $\Delta C_p$ for Grx4 ($-14.5 \pm 3.9 \, \text{cal}\cdot\text{K}^{-1}\cdot\text{mol residue}^{-1}$) and Grx1 ($-23.5 \pm 4.0 \, \text{cal}\cdot\text{K}^{-1}\cdot\text{mol residue}^{-1}$) were within the expected range compared with studies of several monomeric proteins (30). Taken together, these data suggest that Grx4 is well folded and thermally stable, and with similar folding properties as reduced Grx1.

**HED-glutaredoxin Activity of Grx4**—Purified Grx4 was not active in the β-hydroxyethyl disulfide (HED) assay (even in the presence of TrxR), that measures the reduction of the mixed disulfide composed of glutathione and β-mercaptoethanol despite many attempts to find activity (data not shown). The corresponding Grx1 and Grx3 mutants with a CPYS active site served as positive controls. To examine whether the lack of activity could be restored by mutations in the active site and the possible involvement of a cytosolic cofactor, we determined the activity of overexpressed Grx4 and active site dithiol mutants in crude extracts. Strain BL21(DE3)grxA grxB grxC was transformed with plasmids encoding Grx4-CGFS (wild type), Grx4-SGFS, Grx4-CGFC, and Grx4-CPYC. The pET15b plasmid served as control. Crude extract activities of all Grx4 isoforms were equal to that of the control pET15b plasmid (data not shown). All overexpressed mutants were soluble and exhibited strong visible bands (data not shown). Therefore Grx4 cannot reduce the low molecular weight mixed disulfide between glutathione and β-mercaptoethanol. Could it be that the overexpressed protein was not the native Grx4 but a longer molecule? We overexpressed a shorter version of Grx4 starting at methionine 21. The recombinant protein was insoluble (data not shown), therefore not likely to exist under physiological conditions.

**Reduction of Oxidized Grx4**—In the quest for an electron donor for Grx4, we examined the reactivity of GSSG-oxidized Grx4 with TrxR and NADPH (thioredoxin system), or GSH, glutathione reductase, and NADPH (glutaredoxin system). In contrast to the highly similar yGrx5 (31), Grx4 did not react with GSH (Fig. 3A, solid line). Oxidized Grx4 was reduced by TrxR, the reduction corresponding to the consumption of one molecule of NADPH per molecule of Grx4 (Fig. 3A). This most likely reflects a two electron reduction of a disulfide in Grx4. The steady-state kinetics of the reduction of Grx4 by TrxR gave an apparent $K_m$ of 33.3 μM and a $k_{cat}$ of 7.74 s$^{-1}$ (Fig. 3B). Addition of Trx1 (1–2 μM) in the reaction mixture did not affect the reduction rate of Grx4 (data not shown). Grx4 could also be reduced by Grx1 with a stoichiometry of half NADPH molecule per molecule of Grx4 (Fig. 3C). Simultaneous reduction of Grx4 by TrxR and Grx1 resulted in the reduction of 1.5 molecule of NADPH per molecule of Grx4 (data not shown) showing that the disulfides recognized by Grx1 and TrxR were different.

**Determination of Reduced/Oxidized States by Mass Spectrometry**—In parallel, molecular analysis of possible oxidation states of Grx4 was performed by MALDI experiments (Table II). The rational was to estimate the molecular masses of Grx4 with MALDI after treatment with different redox factors (GSSG and DTT), followed by alkylation of free thiols by iodoacetamide (IAM). The three cysteines of Grx4 are tentative targets for glutathionylation and/or disulfide bond formation. Addition of IAM to DTT-reduced Grx4 resulted in mono-, di-, and tri-IAM-alkylated species, thus, all three cysteines could be reduced by DTT (Table II). Oxidation of reduced Grx4 by GSSG followed by IAM treatment under native conditions re-
sulted in monoglutathionylation, but no IAM modification (Table II). In this case, the two remaining cysteines must form a disulfide or be buried in the glutathionylated Grx4. GSSG-oxidized Grx4 subjected sequentially to TrxR treatment and IAM alkylation showed two peaks, one corresponding to a mono-IAM adduct and the other to monoglutathionylation (Table II). This could either imply the reduction of an internal disulfide previously created during oxidation by GSSG, or the reduction of a dimeric Grx4 species. However, only very weak (thus insignificant) Grx4 dimer peaks were observed, the intensities of which did not change after reduction by DTT or TrxR. Thus, TrxR reduced a disulfide of which only one cysteine was accessible to IAM, whereas the enzyme could not reduce the GS-Grx4 mixed disulfide. These data correlate well with the reduction of two thiols of Grx4 by TrxR as determined by spectrophotometry (see previous paragraph).

Alkylation of GSSG-oxidized Grx4 by IAM after Grx1 treatment resulted in the elimination of the monoglutathione adduct, concomitant with the appearance of a double IAM modification. These data suggest that Grx1 could reduce at least one Grx4-SG mixed disulfide species and perhaps another disulfide with one cysteine remaining probably buried in the structure thus, not being accessible to IAM. The stoichiometry of the reduction of GSSG-oxidized Grx4 by Grx1 (Fig. 3C) and the MALDI data point to the direction of an heterogeneous population of Grx4-oxidized species by GSSG. After treating GSSG-oxidized Grx4 with Grx1 and TrxR, and addition of IAM, all three cysteines could be alkylated with some residual disulfide still present (Table II). These data suggest a potential change of Grx4 structure after simultaneous reduction by both Grx1 and TrxR that exposed previously buried thiols to the surface of the protein.

Electron Exchange of Grx4 with Other Proteins—Reduced Grx4 could exchange electrons with oxidized Grx1, Grx1C14S, and Grx3, but not Grx2 and PAPS reductase (Fig. 4). The reactivity of many other proteins such as insulin, and α-glucosidase was also examined in spectrophotometric assays employing GSH/GR or TrxR as electron donors to Grx4 in different pH values but with negative results (not shown). Reduced Grx4 could not reduce glutathionylated BSA in a modified HED assay (data not shown). Grx4 thus has the potential to transfer reducing equivalents but probably to a very specific substrate.

Construction of a Null Mutant for grxD—We tried to construct null mutants for grxD using a method employing a recombinant phage (32) or a temperature-sensitive replication plasmid (33) or the red method (34). We were unable to obtain null mutants for grxD with any of the methods used.

Sandwich ELISA for Grx4—A sensitive sandwich ELISA was developed for Grx4. The detection limit was determined as three times the standard deviation above the blank (27) and was calculated to 0.05–0.1 ng/mg of total cellular protein in the extract. Cross-reactivity of the antibody was detected by adding excess amount of the other glutaredoxins and thioredoxins to see whether they could affect the measured levels of Grx4. The only protein that slightly cross-reacted with the antibodies raised against Grx4 was Grx1. However, the high dilutions of the cell lysates employed for the measurements of Grx4 by ELISA, resulted in such a lowering of the Grx1 contents that its cross-reactivity with antibodies to Grx4 was below the detection limit for Grx1.

Levels of Grx4—Levels of Grx4 were analyzed at the expo-
spot regulating the synthesis of ppGpp, with null mutants for SpoT and RelA being devoid of ppGpp (35). Levels of Grx4 in the wild type strain lowered dramatically at the stationary phase whereas in the wild type strain they increased at the stationary phase (1500–2000 ng/mg) (Fig. 5B). Changes in the levels of Grx4 showed generally the same pattern as those for Trx1, which is regulated by ppGpp, but not RpoS (36, 37). Our findings suggest that transcription of grxD may be regulated by ppGpp at the stationary phase. To confirm this possibility, we measured levels of Grx4 in strains containing different plasmids encoding RelA after inducing expression of the RelA species. A truncated form of RelA (RelA 1–331), which does not elevate ppGpp leads to RelA-dependent accumulation of ppGpp and resulted in a 1.7-fold elevation of Grx4 (data not shown). These results suggest that ppGpp is a positive effector of Grx4.

We next examined whether Grx4 was possibly regulated by ppGpp. Starvation of isoleucine can be induced by addition of valine to the culture medium. Such a treatment leads to RelA-dependent accumulation of ppGpp and resulted in a 1.7-fold elevation of Grx4 (data not shown). These results suggest that ppGpp is a positive effector of Grx4.

Because levels of Grx4 increased upon entry to stationary phase, we examined the levels of Grx4 in null mutants for genes affecting the expression of proteins at the stationary phase of growth. The levels were examined at four different growth phases to reflect growth phase-related changes in transcription. Given the difference of the genetic backgrounds of the different strains studied, we did not compare levels between the different strains but looked at how levels changed within each strain in relation to growth phase.

Guanosine 3’,5’-tetraphosphate (ppGpp) regulates the transcription of many genes at the stationary phase of growth (35). SpoT and RelA are known as the only cellular gene products regulating the synthesis of ppGpp, with null mutants for spoT or relA being devoid of ppGpp (35). Levels of Grx4 in the spoT relA strain lowered dramatically at the stationary phase whereas in the wild type strain they increased at the same growth phase (Fig. 5B). Changes in the levels of Grx4 showed generally the same pattern as those for Trx1, which is regulated by ppGpp, but not RpoS (36, 37). Our findings suggest that transcription of grxD may be regulated by ppGpp at the stationary phase. To confirm this possibility, we measured levels of Grx4 in strains containing different plasmids encoding RelA after inducing expression of the RelA species. A truncated form of RelA (RelA 1–331), which does not elevate ppGpp, levels also did not result in an increase of the levels of Grx4. In contrast, the full-length form (733 amino acids) resulted in a 1.5-fold elevation of Grx4 at 60 min after induction (data not shown), supporting the previous finding that Grx4 is positively regulated by ppGpp.

TABLE II
Redox state of Grx4 thiols as determined by MALDI

| Treatment                                           | MALDI peaks | Corresponding cysteine modification |
|-----------------------------------------------------|-------------|------------------------------------|
| Reduced Grx4 (DTT)                                  | 12,769      | 1 IAM                              |
|                                                     | 12,826      | 2 IAM                              |
|                                                     | 12,879      | 3 IAM                              |
|                                                     | 12,709      | No modification                     |
|                                                     | 13,017      | 1 GS-                              |
| Oxidized Grx4 (GSSG)                                | 12,821      | 2 IAM                              |
|                                                     | 12,761      | 1 IAM                              |
| Oxidized Grx4 (GSSG) plus Grx1                      | 13,018      | 1 GS-                              |
| Oxidized Grx4 (GSSG) plus TrxR                      | 12,757      | 1 IAM                              |
| Oxidized Grx4 (GSSG) plus Grx1 plus TrxR           | 12,887      | 3 IAM                              |

FIG. 4. Electron exchange of Grx4 with other proteins. Oxidized Grx1, Grx1C14S, Grx2, Grx3, and PAPS reductase were incubated with reduced Grx4 (5 μg) for 5 min, alkylated with i-AEDANS, and analyzed by SDS-PAGE. Lane 1, Coomassie staining of Grx1, Grx1C14S, Grx2, Grx3, and PAPS reductase. Lane 2, i-AEDANS fluorescence of the same probes. −, without Grx4; +, with reduced Grx4. Lanes 1, oxidized Grx1, 5 μg; lanes 2, oxidized (glutathionylated) Grx1C14S, 5 μg; lanes 3, oxidized Grx2, 3 μg; lanes 4, oxidized Grx3, 3 μg; lanes 5, oxidized (glutathionylated) PAPS reductase, 5 μg.

FIG. 5. Regulation of Grx4.

A. levels of Grx4 in different E. coli null mutants. Cells were grown in minimal salt medium, and samples were taken at different time points. B. levels of Grx4 in different E. coli null mutants. Cells were grown in minimal salt medium, and samples were taken at late exponential phase (A₁₀₀ 0.7–0.8) (1), early stationary phase (2), after 3 h in stationary phase (3), and finally after 16 h at stationary phase (4). All samples were analyzed by sandwich ELISA, values represent means of duplicates from two independent experiments.

Grx4 was reduced by DTT and oxidized by GSSG as described under “Materials and Methods.” Oxidized Grx4 was treated with TrxR and/or Grx1 and subjected to iodoacetamidation before analysis by MALDI. IAM alkylation resulted in the addition of 57 Da to exposed reduced thiols.
using a plasmid encoding $\sigma^B$ and examining the levels of Grx4 after induction of $\sigma^B$. No significant effect on the levels of Grx4 was observed after induction (data not shown), suggesting that $\sigma^B$ is not contributing to the regulation of transcription of Grx4.

Other factors that did not affect significantly protein levels of Grx4 were osmotic shock (by 0.5 M NaCl) and hydrogen peroxide challenge (1 mM) of wild type strains and null mutants for catalases and OxyR. We assume that $grxD$ does not belong to the OxyR regulon and may not be related to responses against hydrogen peroxide.

**Levels of Grx4 and Iron Content**—Protein levels of Grx4 were measured in a wild type strain and a null mutant for the ferric uptake regulator (Fur) gene ($fur$) in conditions of iron depletion. Fur is a repressor of the transcription of specific genes related with the accumulation of iron. Iron depletion was achieved using 2’,2’-dipyridyl. The steady-state levels of Grx4 were slightly higher in the null mutant for $fur$ compared with the parental wild type strain, suggesting that Fur may be suppressing the transcription of Grx4 (Fig. 6A). The levels of Grx4 were increased upon iron depletion in both the wild type and the null mutant for $fur$ (Fig. 6), although the increase was more pronounced in the $fur^{-}$ strain (Fig. 6B), perhaps reflecting a compensatory involvement of Grx4 in the null mutant in conditions of iron depletion. Levels of Grx4 in the $fur^{-}$ strain returned to normal after 40 min, possibly reflecting the induction of compensatory mechanisms after iron depletion that would allow for the restoration of the levels of Grx4. In summary, our data suggest that Grx4 may be involved in an iron-dependent function that is independent of Fur.

**DISCUSSION**

The glutaredoxin and thioredoxin systems maintain the reduction of cysteine disulfides to give a highly reduced intracellular redox milieu in most living cells. Whereas thioredoxins are reduced by thioredoxin reductase, glutaredoxins are the effectors of GSH. The concept of two separate systems for the reduction of cysteic thiols is quite general in nature with the tripeptide glutathione sometimes replaced by other low molecular weight thiols (39–41). Glutaredoxins can be categorized in three groups, according to their fold and catalytic mechanism (7). The most recently discovered group contains proteins with a monothiol active site with counterparts in Plasmodium falciparum (43). This work describes the characterization of a monothiol glutaredoxin from E. coli for which, due to high homology to yeast monothiol glutaredoxins, we propose the name Grx4 (encoded by $grxD$).

Sequence considerations suggest that Grx4 is representative of the known monothiol glutaredoxins (Fig. 1). The putative active site (CGFS) is identical to that of the known monothiols (Fig. 1). All yeast glutaredoxins have an area of high homology, which includes amino acids 46–132 of yGrx5 and corresponds to the middle of the amino acid sequence of E. coli Grx4 (8–103). In terms of size, Grx4 with 115 amino acids is so far the smallest one in this family of proteins. In comparison, yGrx3 is of 285, yGrx4 of 244, yGrx5 of 151, P. falciparum GLP-1 of 134 (43) and PICOT (42) of 335 amino acids. The elongated N terminus of Grx4 compared with the dithiol glutaredoxins appears to be a characteristic of the monothiol family. The CD spectra show a more pronounced helical fold for Grx4 compared with E. coli Grx1, although the stability measurements suggest that the cores of the two proteins have similar sizes and thermodynamic properties. Other monothiols tend to have an even longer extension than that of Grx4. In the case of yGrx5, part of the N-terminal sequence (a propeptide of 29 amino acids) is not found in the mature form (16). In addition, Grx4 seems to contain a proline residue at position 72 reminiscent of the cis proline of the thioredoxin fold and a glycine pair (Gly$^{82}$–Gly$^{83}$ for Grx4) close to its C terminus. Both features are present in the sequences of Grx1 and Grx3. The first feature is required for interactions between GSH and glutaredoxins (9), whereas the second is part of the surface that surrounds the active site of Grx3 (10) and has very close proximity to the glutamic acid of bound GSH (44). In yGrx5 the GG pair is considered necessary for the structural integrity of the cleft required for GSH binding (45).

Grx4 is the first glutaredoxin that, after GSSG oxidation, acts as a substrate for E. coli TrxR. This result is in itself remarkable, because E. coli TrxR is highly substrate-specific and will not catalyze reduction of the active site disulfide in Grx1, Grx2, or Grx3 (1). In comparison to the known substrates for E. coli TrxR (Trx1, Trx2, and NrdH), Grx4 had a higher but physiological $K_m$ (33 $\mu M$), whereas the $k_{cat}$ (8 s$^{-1}$) was similar to those of the other redoxins. The mitochondrial human Grx2 may also accept electrons from TrxR, but from the mammalian TrxR, which has broad substrate specificity (46). The reduction of human Grx2 by the mammalian TrxR has a $K_m$ value of 22 $\mu M$ (47), similar to that of Grx4. A summary of the steady-state
kinetic parameters for the interaction of different redoxins with TrxR is shown in Table IV. The spectrophotometric measurements together with the MALDI data suggest the reduction of one intramolecular disulfide by TrxR and one mixed disulfide between the remaining thiol and the -SG moiety by Grx1. The proposed reduction of the oxidized thiols of GSSG-oxidized Grx4 by sequential TrxR and Grx1 treatment is portrayed in Fig. 7. A structural interpretation of the MALDI data is presented in the accompanying paper (18).

Despite its pronounced sequence similarity to other glutaredoxins and its well folded properties as shown by CD, Grx4 was not active in the HED assay in which known dithiol E. coli glutaredoxins are active (6, 33). The substrate for this assay is the mixed disulfide between β-mercaptoethanol and a GS-moiety. To elaborate further on the substrate specificity of Grx4, mutants having dithiol (CGFC and CPYC) active sites were overexpressed, and their activities were measured. These mutants were also inactive in the diagnostic for glutaredoxin activity HED assay, even in crude extracts. yGrx5 was also not active in the HED assay or the assays with dehydroascorbate reductase or glutathione peroxidase (31). One reason for this lack of activity could be the very low reduction rates of yGrx5 with glutathione (31). Previously reduced yGrx5 could deglutathionylate carbonic anhydride, but its physiological substrate as well as its physiological electron donor remain unknown (31). Reduction of Grx4 by TrxR is the first evidence of a physiological electron donor for the monothiol glutaredoxins. Reduced Grx4 itself could exchange electrons with the intracellular disulfides of Grx1 and Grx3 showing that the protein can undergo redox reactions and potentially can form part of an electron chain. However, Grx4 was not reacting with the disulfide of Grx2 or the GS-mixed disulfides of Grx1C14S or PAPS reductase. Given the observed inability of Grx4 to reduce different substrates at measurable rates when in catalytic amounts, it is likely that Grx4 has very high substrate specificity.

Grx4 is highly abundant in the cell, with levels up to 2 µg/mg of total soluble protein at the stationary phase of growth. Like the other three glutaredoxins of E. coli (25), Grx4 was up-regulated in mutants lacking the thioredoxin system. As is the case for Grx2 and Trx1 (36, 37), Grx4 was up-regulated at the stationary phase of growth with an almost 3-fold increase, reflecting a vital yet unknown function of Grx4 in the stationary phase. The regulation of Grx4 thus differs from that of the yeast monothiol glutaredoxins, which were all at their maximum expression during the exponential phase of growth and their mRNA levels decreased under detection limits at the stationary phase (15). E. coli possesses a remarkable ability to adapt and survive under the harsh conditions of the stationary phase (15). Their mRNA levels decreased under detection limits at the mum expression during the exponential phase of growth and yeast monothiol glutaredoxins, which were all at their maximum

### Table IV

| Redoxin<sup>a</sup> | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | Substrate<sup>b</sup> | Reference |
|---------------------|------|----------|---------------|------------------------|-----------|
| Trx1                | 1.9  | 11.3     | $6.3 \times 10^6$ | DTNB                   | (58)      |
| Trx2                | 2.4  | 12.8     | $5.4 \times 10^6$ | DTNB                   | (58)      |
| Grx4                | 33.3 | 8        | $0.24 \times 10^4$ | Direct                 | This work |
| hGrx2               | 22   | 14       | $0.64 \times 10^4$ | Direct                 | (47)      |
| hTrx1               | 2.5  | 50       | $20 \times 10^4$  | Insulin, DTNB          | (59)      |

<sup>a</sup> Trx1, Trx2, Grx1, and Grx4 were from E. coli; hGrx2 and hTrx1 were from human.

<sup>b</sup> The used substrates were 5,5'-dithiobis-(2-nitrobenzolic acid) (DTNB), insulin (19), or preparations of fully oxidized Grx1, Grx4, and hGrx2 (direct).

Grx4 is regulated at the stationary phase by ppGpp, but not α<sup>54</sup>. Based on their up-regulation pattern, glutathione reductase, Grx4, Grx2, and Trx1 may be particularly important redox-active proteins for cells at the stationary phase of growth.

The sensor for free iron concentration in E. coli and other Gram-positive and -negative bacteria is the Fur protein. Fur binds to Fe<sup>2+</sup> ions, and the complex can bind to consensus sequence (Fur box) upstream genes related to iron uptake such as the iron transport systems of E. coli. The binding of Fur-Fe<sup>2+</sup> results in repression of transcription (50). A tight control of iron uptake is essential to avoid excess of free iron that could generate free radicals via Fenton chemistry. Grx4 levels were slightly higher at the fur<sup>−</sup> strain suggesting a potential involvement of Fur in the transcription of Grx4. Depletion of iron caused a dramatic elevation of the levels of Grx4, especially in the case of the fur<sup>−</sup> strain indicating that Fur is not the main regulator of the transcription of Grx4. Our data suggest that the protein may be involved in pathways using iron. In yeast, elimination of yGrx5 prevented respiratory growth and caused accumulation of free iron in the cell (16).

Levels of Grx4 did not change after treatment with hydrogen peroxide, or in null mutants for catalases or OxyR, suggesting that grxD is not part of the OxyR regulon (as grxA is (51)), and is not involved in responses against hydrogen peroxide. Yeast monothiol glutaredoxins (particularly yGrx5), showed no increase in their transcription either, but rather a decrease after exposure to hydrogen peroxide (15), but protected cells against oxidative and osmotic stress, since the relevant null mutants were much more sensitive (15). The antioxidant response in E. coli is complicated, and one could not exclude an antioxidant role for Grx4. For example E. coli Grx2 levels decreased after exposure to hydrogen peroxide, but were significantly up-regulated in catalase deficient strains (25). Furthermore, null mutants for grxB had high levels of carbonylated proteins in their cytosols after exposure to hydrogen peroxide compared with wild type cells (25, 52). Apparently the levels of a redox-active protein after exposure to a certain oxidant reflect the sum of metabolic processes where the redoxin participates.
The existence of a monothiol glutaredoxin in *E. coli*, confirms the ubiquity of these enzymes in eukaryotes and prokaryotes. The essential role of the monothiol glutaredoxins has clearly been demonstrated for yeast (15), but also for *E. coli* Grx4 (17). Grx4 was identified as an unknown essential gene, by a genetic footprinting technique, for a genome-wide assessment of genes required for robust aerobic growth of *E. coli* in rich media (17). Our efforts to make such a null mutant have been fruitless, despite the variety of techniques used. Because the grxD gene is essential, it would be the first component of the thioredoxin/glutaredoxin system that has such a property. Obviously the function of Grx4 may then involve an aspect not overlapping in vivo in E. coli metabolism. The NMR solution structure of Grx4 is presented in the accompanying paper (18).

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Aristi Potamitou Fernandes, Malin Fladvad, Carsten Berndt, Cecilia Andrésen, Christopher Horst Lillig, Peter Neubauer, Maria Sunnerhagen, Arne Holmgren and Alexios Vlamis-Gardikas

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