Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9813–9817, October 1994
Biochemistry

Two additional glutaredoxins exist in *Escherichia coli*: Glutaredoxin 3 is a hydrogen donor for ribonucleotide reductase in a thioredoxin/glutaredoxin 1 double mutant

(glutathione–disulfide oxidoreductase/thioltransferase/redox-active disulfides/enzyme purification/DNA biosynthesis)

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Communicated by Peter Reichard, June 23, 1994

ABSTRACT **Thioredoxin (Trx) and glutaredoxin (Grx1) are hydrogen donors for ribonucleotide reductase, the key enzyme for deoxyribonucleotide biosynthesis. The viability of a double mutant lacking both Trx and Grx1 implies the presence of a third, unknown hydrogen donor. This paper reports the purification and characterization of two proteins with glutaredoxin activity (using hydroxyethyl disulfide as a substrate) from an *Escherichia coli* mutant lacking Trx and Grx1 (Δ*trxA*, *grx*::*kan*). Affinity chromatography was used to bind glutaredoxin on a glutathione-containing thiol-Sepharose column. The molecular weight of Grx2, 27,000, was atypical for glutaredoxins, whereas Grx3 had a molecular weight of 10,000. Amino acid sequence analysis revealed novel structures with putative active sites typical of glutaredoxins: Cys-Pro-Tyr-Cys. The proteins are therefore referred to as Grx2 and Grx3. The low hydrogen donor activity for ribonucleotide reductase in the crude extract was recovered in the purification of Grx3, whereas Grx2 was inactive. As a hydrogen donor for *E. coli* ribonucleotide reductase, Grx3 showed approximately the same K_m value (0.35 μM) as Grx1, whereas its V_{max} value was only 5% that of Grx1. The combination of the Grx3 hydrogen donor activity and a 25-fold induction of ribonucleotide reductase activity in a Δ*trxA*, *grx* double mutant provides an explanation for its viability and deoxyribonucleotide biosynthesis. The physiological functions of Grx2 and Grx3 remain to be determined.**

Thioredoxin and glutaredoxin were discovered separately as hydrogen donors for ribonucleotide reductase (1, 2), the enzyme catalyzing deoxyribonucleotide biosynthesis (3). Both thioredoxin and glutaredoxin contain in the oxidized form two cysteine residues forming a 14-member disulfide ring in their active site, with sequences Cys-Gly-Pro-Cys in thioredoxin and Cys-Pro-Tyr-Cys in glutaredoxin. Oxidized thioredoxin is reduced by NADPH and thioredoxin reductase, whereas glutaredoxin is reduced by glutathione (GSH), which is kept in a reduced form by NADPH and GSH reductase (4). Also adenosine 3′-phosphate 5′-phosphosulfate reductase (an enzyme in the sulfate reduction pathway) and methionine sulfoxide reductase require thioredoxin or glutaredoxin (5).

Thioredoxin and glutaredoxin have been assumed to be required as hydrogen donors for ribonucleotide reductase, which is essential for DNA synthesis. Yet an *Escherichia coli* double mutant (6) lacking thioredoxin and glutaredoxin (Grx1) could be constructed. The best-growing mutant that was selected (A410) had acquired an extra mutation in the sulfate assimilation pathway (*cysA*), presumably to avoid toxic accumulation of adenosine 3′-phosphate 5′-phosphosulfate. Thus, the mutant was not viable on minimal medium unless a source of reduced sulfur was added. This indicates that thioredoxin and glutaredoxin (Grx1) are not essential for deoxyribonucleotide biosynthesis but are essential for the reduction of sulfate (7), implying the presence of a third, unknown hydrogen donor system for ribonucleotide reductase in *E. coli*.

The isolation of a *Corynebacterium nephridii* thioredoxin (8) and a rabbit bone marrow glutaredoxin (9) and thioredoxin (10) that do not react with their homologous ribonucleotide reductases has cast further doubts on the general involvement of thioredoxin and glutaredoxin as the only hydrogen donors for ribonucleotide reductase. Furthermore, immunohistochemical studies show no clear correlation of the distribution of thioredoxin and ribonucleotide reductase in most rat tissues, indicating that thioredoxin is not the physiological hydrogen donor (11).

It was observed when *E. coli* glutaredoxin (Grx1) was purified that this protein had activity as a GSH–disulfide oxidoreductase. But, 98% of such activity in the crude extract was of unknown origin (12). The activity was measured as the enzymatic reduction of the mixed disulfide between the prototype substrate β-hydroxyethyl disulfide (HED) and GSH at the expense of NADPH (recorded at 340 nm) by using the HED assay of Holmgren (12). The HED assay is based on the following reactions (where XSSX is HED):

$$\mathrm{GSH + XSSX \rightarrow GSSX + XSH} \quad [1]$$

$$\mathrm{GSH + GSSX \xrightarrow{Glutaredoxin} GSSG + XSH} \quad [2]$$

$$\mathrm{GSSG + NADPH + H^+ \xrightarrow{GSH\ reductase} 2\ GSH + NADP^+,} \quad [3]$$

giving a net reaction

$$\mathrm{NADPH + H^+ + XSSX \rightarrow NADP^+ + 2\ XSH}$$

We have now purified the enzymes responsible for this activity to homogeneity, resulting in the identification of Grx2 and Grx3. We present evidence that Grx3 is likely to be the substitute for thioredoxin and glutaredoxin (Grx1) in the double mutant and, in combination with a large induction of ribonucleotide reductase activity, forms a system sufficient for the supply of deoxyribonucleotides.

MATERIALS AND METHODS

Materials. [^{3}H]CDP was from Amersham. HED was from Aldrich. Phenylmethylsulfonyl fluoride was from Boehringer

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Abbreviations: GSH, glutathione; HED, β-hydroxyethyl disulfide.

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Mannheim. DEAE-cellulose (DE-52) anion-exchanger was from Whatman. NAP-5 prepacked columns, activated thiol-Sepharose, and Sephadex G-50 were from Pharmacia–LKB. Diaflo YM3 membranes were from Amicon. SpectraPor membrane tubing was from Spectrum Medical Industries.

Yeast GSH reductase was from Sigma. *E. coli* ribonucleotide reductase subunits R1 and R2 in equal proportions were a gift from Professor Britt-Marie Sjöberg (Department of Molecular Biology, Stockholm University). The mixture was stored as aliquots in −70°C at a concentration of 10 mg/ml. *E. coli* thioredoxin, thioredoxin reductase (13), and glutaredoxin (Grx1) (14) were homogeneous preparations from this laboratory. Inhibitory antibodies against *E. coli* glutaredoxin (Grx1) were from a rabbit antiserum (15).

The strain used for purification (UC647) was *E. coli* K-12 (Δ*trxA*, *grx*::*kan zbi*::Tn*10*, pKM101) (16). This strain is a derivative that grows on minimal-medium plates, but not in liquid minimal medium. As wild-type strain we used UC1101 (17), which is the parental wild type of UC647.

Large-Scale Growth of Cells. A 1-ml preculture of UC647 in FB medium (18) with 0.5% glucose and kanamycin at 50 μg/ml was added to a glass bottle containing 0.5 liter of the same medium. This culture was grown overnight and then used to inoculate 20 liters of LB medium in an automatic fermentor (Microferm; New Brunswick Scientific) with the addition of 0.5% glucose and kanamycin at 50 μg/ml. The culture was grown aerobically to a final OD_{650} of 1.5 and then harvested by centrifugation. The bacterial cell pellet was stored at −70°C until used.

GSH-Disulfide Oxidoreductase Assay. The assay was performed as described by Holmgren (12), measuring the reduction of HED by GSH at the expense of NADPH by monitoring absorbance at 340 nm. A standard of purified *E. coli* Grx1 was used in each experiment as a positive control. One unit was defined as the oxidation of 1 μmol of NADPH per min.

Protein Concentration. This was determined as described by Bradford (19).

Purification of Grx2 and Grx3. Unless otherwise noted, all operations were performed at 4°C. The columns used in the purification were used solely in this project, to avoid contamination. Three aliquots of 100 μl were taken from each step in the purification, stored at −70°C, and analyzed in parallel with the various assays described in this section.

Frozen cells (40 g) were thawed and lysed with lysozyme (0.2 mg/ml) by incubation for 1 hr in 260 ml of 50 mM Tris Cl, pH 7.5/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride on ice. The lysate was frozen and thawed and sonicated thoroughly. A crude extract (extract 1) was prepared by centrifugation at 15,000 × *g* for 30 min. Nucleic acids were precipitated by slow addition of 0.1 volume of freshly prepared 5% streptomycin sulfate solution and the precipitate was discarded after centrifugation.

The supernatant fraction (extract 2) was saturated to 40% with solid ammonium sulfate and then stirred for 1 hr. The precipitate was discarded by centrifugation. Ammonium sulfate was then added to the resulting supernatant to 95% saturation and the pelleted protein was collected by centrifugation, dissolved in 20 mM Tris Cl, pH 9.5/1 mM EDTA, and dialyzed extensively against the same buffer. The extract after dialysis (extract 3) was loaded onto a 300-ml column of DE-52 anion exchanger equilibrated with 20 mM Tris Cl, pH 9.5/1 mM EDTA. The column was then washed with 1 liter of 10 mM Tris at pH 9.5. The material was eluted from the column with 2 liters of 50 mM Tris Cl (pH 8.0). The active fractions were concentrated to 5 ml by ultrafiltration on a YM3 ultrafiltration membrane (molecular mass cutoff, 3 kDa), incubated with 10 mM dithiothreitol for 30 min at 37°C, and applied to a column (100 cm × 3 cm^2) of Sephadex G-50 equilibrated with degassed 20 mM Bistris Cl, pH 6.0/1 mM EDTA. Two activity peaks were obtained and pooled separately [extracts 5a (Grx2) and 5b (Grx3)]. Each was immediately applied, at a slow flow rate (0.2 ml/min), onto two identical 10-ml columns of activated thiol-Sepharose equilibrated with 20 mM Bistris Cl, pH 6.0/1 mM EDTA. The columns were washed extensively with the same buffer, and protein was eluted with 30 ml of 5 mM GSH/50 mM Tris Cl, pH 8.0. Active fractions were concentrated by ultrafiltration on a Diaflo YM3 membrane and transferred to 50 mM Tris (pH 8.0) by repetitive additions of this buffer.

Material for amino acid sequence analysis was prepared by C_4 reverse-phase HPLC. Both proteins retained some activity in the HED assay after the HPLC step and could thus be identified.

Bacterial Crude Extracts for Ribonucleotide Reductase Determinations. Wild-type (UC1101) and mutant (UC647) bacteria were grown aerobically in LB medium and harvested in exponential phase by centrifugation. Frozen cells were sonicated with 4 times their weight of 50 mM Tris Cl, pH 7.5/1 mM EDTA. Cell-free extracts were prepared by centrifugation at 10,000 × *g* for 10 min and the supernatants were immediately used for ribonucleotide reductase activity determinations.

Determination of Ribonucleotide Reductase Activity. This was assayed as described by Thelander *et al.* (20) and Holmgren (12, 21). Activity was measured as the conversion of [^{3}H]CDP to [^{3}H]dCDP. Ribonucleotide reductase activity in crude extracts was determined in the presence of an excess of the thioredoxin system (12 μM thioredoxin, 0.2 μM thioredoxin reductase, and 2.0 mM NADPH). Incubations were at 37°C for 20 min.

Hydrogen Donor Activity Determination. Hydrogen donor activity in the extracts from the purification was determined in the presence of 10 μg of reduced and desalted ribonucleotide reductase (21) by using the ribonucleotide reductase assay described above. As reductants, either 4.0 mM GSH, 1.0 mM NADPH, and glutathione reductase at 0.01 mg/ml or 1 mM dithiothreitol were used in separate experiments.

The determination of hydrogen donor activity in crude extracts of UC647 was done with subtraction of the high endogenous ribonucleotide reductase activity.

In the assays, at least three different concentrations of extract were used and all experiments were performed at least twice. To rule out the presence of Grx1, assays were also done in the presence of inhibitory antibodies against Grx1. A standard of homogeneous Grx1 was used in each assay as positive control.

Thioredoxin Determinations. These were performed by measuring the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of thioredoxin reductase and NADPH (22) upon the addition of heated (85°C for 5 min) crude extracts. Assays were also performed with additions of heated crude extracts to 2.0 mM NADPH, 0.2 μM thioredoxin reductase, and 10 μg of ribonucleotide reductase. In both types of assays a standard of homogeneous *E. coli* thioredoxin was used as positive control.

SDS/PAGE. Electrophoresis was performed with the Phast gel system of Pharmacia, employing SDS/8–25% polyacrylamide gradient gels with Coomassie staining.

N-Terminal Amino Acid Sequences of Grx2 and Grx3. The N-terminal sequences of [^{14}C]carboxymethylated Grx2 and Grx3 were determined with a MilliGen Prosequencer 6600.

RESULTS

Purification of Grx2 and Grx3. Initial attempts to purify the hydrogen donor for ribonucleotide reductase in Δ*trxA*, *grx* cells were done with the original strain A410 (6). As we had difficulties identifying a hydrogen donor in A410, we started working with a newly constructed strain, *E. coli* K-12 UC647

Table 1. Purification of *E. coli* Grx2 and Grx3

Step	Extract no.	HED activity, units	Specific activity, units/mg
Crude extract	1	2190	0.73
Streptomycin sulfate supernatant	2	2145	0.92
DE-52 pool	4	1370	29
Sephadex G-50			
pool 1 (Grx2)	5a	1010	48.9
pool 2 (Grx3)	5b	214	42.5
Thiol-Sepharose			
Of pool 1 (Grx2)	6a	918	287
Of pool 2 (Grx3)	6b	135	175

Starting material was 42 g of UC647 cells. Details are given in *Materials and Methods*. GSH–disulfide oxidoreductase activity was determined by the HED assay in aliquots stored frozen at −70°C.

(16). This strain differs from A410 by its viability on minimal medium plates. On the assumption that a hydrogen donor might be induced to higher levels, we chose to work with UC647.

A reproducible purification of two independent GSH-disulfide transhydrogenases from UC647 was developed (Table 1).

A 40–95% ammonium sulfate precipitation showed only one peak of activity on the DE-52 column, eluted with 50 mM Tris (pH 8.0). The active fractions were concentrated by ultrafiltration to 5 ml and incubated with 10 mM dithiothreitol and loaded onto a Sephadex G-50 column. The incubation with dithiothreitol was done in order to avoid formation of dimers of Grx3 and to have reduced and desalted proteins for the thiol-Sepharose chromatography in the following step. Two pools of activity resulted from the Sephadex G-50 chromatography (Grx2 and Grx3). Both were further purified on two separate columns of activated thiol-Sepharose. To ensure a high degree of binding of Grx2/Grx3, the sample applications were done at pH 6.0 and at a low flow rate.

Grx2 was found to be >90% pure after the thiol-Sepharose step as judged by SDS/PAGE and on analytical C_4 reverse-phase HPLC. Grx3 was 50% pure as judged by the same criteria. The total recovery of activity units was 48%. Specific activities of 287 units/mg for pure Grx2 and 350 units/mg for pure Grx3 were calculated and have been used for determination of K_m values and estimation of intracellular concentrations.

Homogenous material of Grx2 and Grx3, for amino acid sequence analysis was obtained by preparative C_4 reverse-phase HPLC using a gradient from 0.1% trifluoroacetic acid in water to 75% acetonitrile/0.1% trifluoroacetic acid. Both Grx2 and Grx3 retained some of their respective HED activities and could be identified in the chromatogram. Both proteins were eluted at around 35% acetonitrile.

Ribonucleotide Reductase Levels of Wild-Type and Mutant Crude Extracts. During our initial determinations of hydrogen donor activity in crude extracts of the double mutant, we observed high amounts of ribonucleotide reductase activity. To determine to what extent ribonucleotide reductase was induced, saturating amounts of thioredoxin (12 μM), thioredoxin reductase (0.2 μM), and NADPH (2 mM) was added to crude extracts of the mutant and the wild type to determine ribonucleotide reductase activity. The mutant contained much higher (25-fold) levels of ribonucleotide reductase activity than the wild type. Similar results were obtained with Western blot analysis using an antibody against subunit R1 of *E. coli* ribonucleotide reductase (data not shown).

Hydrogen Donor Activity in Extracts from the Purification of Grx2 and Grx3. The low hydrogen donor activity of the crude extract was recovered in the purification of Grx3, demonstrating that Grx3 is the relevant hydrogen donor for

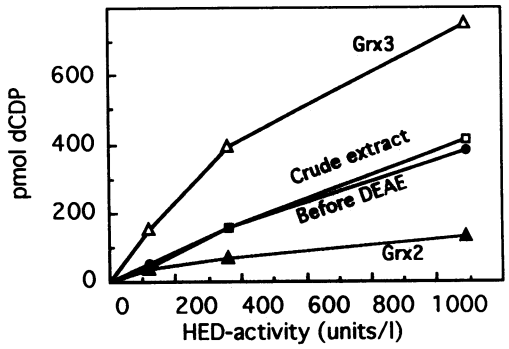

FIG. 1. Hydrogen donor activity of various extracts from the purification of Grx2 and Grx3 (see Table 1) with *E. coli* ribonucleotide reductase plotted against HED activity in the presence of 4 mM GSH, 1 mM NADPH, and excess GSH reductase. Incubation time was 20 min. □, Crude extract (extract 1); ●, before DEAE-cellulose (DE-52) (extract 3); ▲, Grx2 (extract 6a); △, Grx3 (extract 6b).

ribonucleotide reductase in the double mutant (Fig. 1). The activity of Grx3 was not inhibited by addition of antibodies against Grx1, thereby excluding the possibility of contamination by Grx1. This also shows that Grx3 is structurally distinct from Grx1.

The crude extracts of the UC647 mutant were also analyzed to determine whether the hydrogen donor activity was stimulated by the addition of thioredoxin reductase and NADPH (Table 2). No stimulation above the value for NADPH alone was observed, indicating that thioredoxin reductase is not involved in the hydrogen donor system in the mutant. Addition of GSH, GSH reductase, and NADPH resulted in a further stimulation, supporting our view that the hydrogen donor activity in the double mutant is GSH-dependent.

Molecular Size of Grx2 and Grx3 and Estimated K_m and V_{max} for Grx3 with Ribonucleotide Reductase. The size of Grx2 and Grx3, as determined by SDS/PAGE, was 27 kDa (Fig. 2, lane 3) and 10 kDa (lane 4), respectively, similar to Grx1 (lane 2). Dimers of Grx3 were also found (lane 5) when dithiothreitol was not added to the sample.

Assays of Grx3 with ribonucleotide reductase showed Michaelis–Menten kinetics, and K_m and V_{max} values were calculated by using a specific activity of 350 units/mg in the HED assay for pure Grx3 and a molecular weight of 10,000. The activity of Grx3 was much lower than that of Grx1 (Fig. 3). The V_{max} was only 5% of that for Grx1, but the K_m value was 0.35 μM—similar to that of Grx1 (0.13 μM) (21).

N-Terminal Amino Acid Sequences of Grx2 and Grx3. N-terminal amino acid sequence analysis of Grx2 and Grx3 revealed unambiguous sequences with putative active sites

Table 2. Assay of deoxyribonucleotide synthesis in a crude extract of *E. coli* UC647

Addition	dCDP, pmol
None	120
5 mM NADH	180
5 mM NADPH	200
5 mM NADPH, 4 mM GSH, and 1.5 μg of GSH reductase	370
5 mM NADPH and 2.5 μg of thioredoxin reductase	180
5 mM NADPH, 2.5 μg of thioredoxin reductase, and 10 μM thioredoxin	13,000

Cells were sonicated and a supernatant was obtained by centrifugation. An aliquot containing 200 μg of protein was used with [^{3}H]CDP.

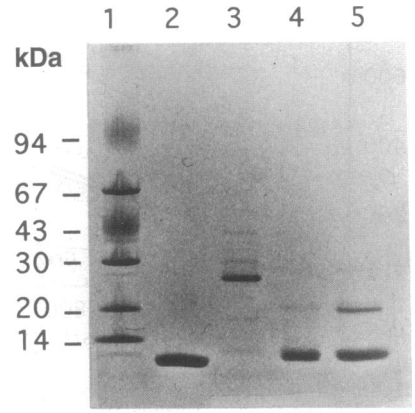

FIG. 2. SDS/PAGE. Lane 1, molecular size markers; lane 2, Grx1; lane 3, Grx2 (extract 6a); lane 4, Grx3 (extract 6b, further purified by HPLC); lane 5, Grx3 with dithiothreitol omitted from the sample buffer, showing Grx3 dimer.

typical of glutaredoxins, Cys-Pro-Tyr-Cys (Table 3). The rest of the Grx2 and Grx3 sequence determined was distinct from that of Grx1.

DISCUSSION

The viability of a *trxA, grx* double mutant has revealed that thioredoxin and glutaredoxin are not essential for deoxyribonucleotide biosynthesis (6) but are needed for sulfate reduction (7). The strain used in this project (UC647) differs from the previously characterized double mutant A410 by its ability to grow on minimal-medium plates, without the addition of a source of reduced sulfur. Conceivably, the source of reducing equivalents in this mutant may be any of the following: NADPH/thioredoxin reductase, NADPH/GSH/GSH reductase, or NADH/lipoamide dehydrogenase. When the double mutant A410 was characterized (7), an NADPH-linked system for ribonucleotide reduction in high-speed supernatants was noted. The finding that addition of NADPH/GSH/GSH reductase (but not NADPH/thioredoxin reductase) to crude extracts of the mutant gave an enhanced production of deoxyribonucleotides compared with NADPH or NADH alone (Table 2) prompted us to focus on this system.

E. coli contains high amounts of GSH–disulfide oxidoreductase activity of unknown origin (12). We have purified the

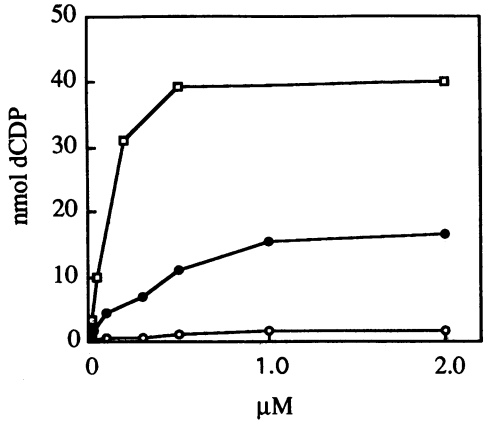

FIG. 3. Comparison between *E. coli* Grx1 and Grx3 (extract 6b) as hydrogen donors for ribonucleotide reductase (20 μg) in the presence of 4 mM GSH, 1 mM NADPH, and excess GSH reductase. Incubation time was 20 min. □, Grx1; ○, Grx3; ●, Grx3 (values multiplied by a factor of 10).

Table 3. N-terminal amino acid sequences of the three different *E. coli* glutaredoxins aligned after the identical active sites (CPYC, underlined)

Protein	Sequence
Grx1	MQTVIFGRSGCPYCVRAKDL
Grx2	MKLYIYDHCPYCIKARMI
Grx3	ANVEIYTKETCPYCHRAKAL

enzymes responsible for this activity in high yield and to near homogeneity, resulting in identification of the two proteins called Grx2 and Grx3. A method for affinity chromatography of glutaredoxins on activated thiol-Sepharose was developed. The binding of Grx2 and Grx3 on this column is likely to be as mixed disulfides between glutaredoxin and the GSH spacer arms of thiol Sepharose. Reduced and desalted Grx2 and Grx3 were applied to activated thiol-Sepharose columns at pH 6.0. The rational behind this was to have only the more N-terminal active-site cysteine in the thiolate form, due to the different pKa values of the active-site thiols typical of glutaredoxins. The more N-terminal active-site cysteine of glutaredoxins has a pKa value that is unusually low (23), below 5 for *E. coli* Grx1 (O. Björnberg and A.H., unpublished work), whereas the more C-terminal active-site cysteine has a normal pKa value, around 9 (24). Thus, at pH 6.0 the more N-terminal active-site cysteine would be in the thiolate form, ready to initiate a nucleophilic attack on the glutathione-2-pyridyl disulfide of the thiol-Sepharose medium, whereas the C-terminal active-site cysteine would be protonated and inactive and thus unable to promote reoxidation of the active site.

Grx3 has a molecular mass typical of glutaredoxins (10 kDa) whereas Grx2 is an atypical glutaredoxin due to its higher molecular mass (27 kDa). As a hydrogen donor for *E. coli* ribonucleotide reductase, only Grx3 showed activity. Furthermore the hydrogen donor activity of the crude extract was recovered in the purification of Grx3. The intracellular concentrations of Grx2 and Grx3 in the double mutant can be calculated to be 5 μM and 2.4 μM respectively, based on their molecular weights, specific activities, and ratios after separation by Sephadex G-50 chromatography (Table 1). The intracellular concentration of Grx1 in wild-type cells was previously estimated to be 0.2 μM (12). As discussed above, the total activity of GSH–disulfide oxidoreductase activity in *E. coli* is about 50-fold higher than what can be attributed to Grx1 (specific HED activity, 220 units/mg) (14). Provided that the concentration of Grx2 and Grx3 in wild-type cells is similar to that in the double mutant, the relative contributions to the HED activity would be Grx1, 0.9%; Grx2, 81.4%; and Grx3, 17.6%; explaining the missing 98–99%. With a K_m value of 0.35 μM for ribonucleotide reductase and a concentration of 2.4 μM in the double mutant, Grx3 would be working at its V_{max} level in the cell. Even though Grx3 is quite inefficient as a hydrogen donor (V_{max} only 5% that of Grx1), the combination of Grx3 and the very high levels of ribonucleotide reductase in the mutant provides an explanation for its viability (16). Grx3 coupled to the cellular GSH is thus likely to be the postulated third hydrogen donor system, in the absence of thioredoxin and the classic glutaredoxin (Grx1). Under normal conditions, it is obvious that Grx3 must have other functions in the cell. This finding and the high level of ribonucleotide reduction seen only in *trxA, grx* double mutants and not in *trxA* or *grx* single mutants (16) support the importance of glutaredoxin (Grx1) and thioredoxin as *in vivo* hydrogen donors for ribonucleotide reductase.

A glutaredoxin (thioltransferase) has been purified from human erythrocytes (25, 26). Since erythrocytes do not synthesize DNA, glutaredoxins must have important func-

tions in the cell other than as hydrogen donors for ribonucleotide reductase. These functions may be of a specific nature, such as supplying reducing equivalents to a specific enzymatic reaction, or of a general nature, protecting cells from oxidative stress together with GSH. Studies of Grx2 and Grx3 will perhaps shed light on these other functions of glutaredoxins.

The size of thioredoxins and glutaredoxins has enabled the determination of their structures to high resolution by NMR and by x-ray crystallography. This makes thioredoxins and glutaredoxins among the best-characterized proteins and has demonstrated a common fold despite low amino acid sequence homology (27). The isolation of Grx3 adds one more member to the glutaredoxin family of proteins with a M_r of 10,000–12,000. The isolation of Grx2, a glutaredoxin with atypical molecular weight, might provide new insights from a structure–function point of view. As seen from Table 3, the active site of Grx2 is located in the same relative position as in other glutaredoxins. This leaves open the question of the potential functions of the C-terminal extension of the protein accounting for 17 kDa of its molecular mass. Whether Grx2 is represented in higher organisms than prokaryotes will be of great interest.

We acknowledge help from Prof. Hans Jörnvall in N-terminal amino acid sequencing, Prof. Britt-Marie Sjöberg for gifts of ribonucleotide reductase, Monica Lindell for generous help with illustrations, and Jack Andrzejewski for fermentor growth of bacteria. This investigation was supported by grants from the Swedish Medical Research Council (13X-3529) and the Swedish Cancer Society (961), the Inga-Britt och Arne Lundbergs Stiftelse, the Knut och Alice Wallenberg Stiftelse, and the Karolinska Institute. A.M.-V. was supported by a fellowship from the Ministerio de Educación y Ciencia, Spain, and by a short-term European Molecular Biology Organization fellowship.

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