Reactivity of glutaredoxins 1, 2 and 3 from *Escherichia coli* and protein disulfide isomerase towards glutathionyl-mixed disulfides in ribonuclease A

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Abstract **We have examined the activity of protein disulfide isomerase (PDI) and glutaredoxin (Grx) 1, 2 and 3 from *Escherichia coli* to catalyze the cleavage of glutathionylated ribonuclease A (RNase-SG) by 1 mM GSH to yield reduced RNase. Apparent K_m values for RNase-SG were similar, 2–10 μM, for Grx 1, 3 and PDI but Grx 1 and Grx 3 showed 500-fold higher turnover numbers than PDI. The atypical Grx 2 also catalyzed deglutathionylation by GSH, but had higher K_m and apparent turnover number values compared to the two classical Grx. Refolding of RNase in a glutathione redox buffer was catalyzed by PDI. However, it could be measured only after a characteristic lag phase that was shortened by all three *E. coli* Grxs in a concentration-dependent manner. A role of the glutaredoxin mechanism in the endoplasmic reticulum is suggested.**

Key words: Glutaredoxin; Protein disulfide isomerase; Glutathione; Endoplasmic reticulum; Mixed disulfide

1. Introduction

Formation of native protein disulfide bonds is catalyzed by protein disulfide isomerase (PDI) and possibly several isoenzymes, all with thioredoxin domains, in the lumen of the endoplasmic reticulum (ER) [1]. Both GSH and GSSG are present in the ER and at a ratio which should promote disulfide bond formation [2] but the role of GSSG as the oxidant of protein thiols in the ER is not clear [3]. In vitro, PDI and a redox buffer of GSH/GSSG permit efficient folding and disulfide bond formation in several model proteins, despite the fact that PDI appears to have no apparent specificity for glutathione [4]. In contrast *Escherichia coli* glutaredoxins (Grx) 1, 2 and 3 catalyze GSH-dependent oxidoreduction reactions, for example the reduction of the mixed disulfide between β-mercaptoethanol and glutathione [5]. Grx 1 and 3 (9 kDa) have similar overall structures, active sites and activities in a standard reduction of 2-hydroxyethyl disulfide by GSH [6] but Grx 1 has a 35 mV lower redox potential compared to Grx 3 [7]. Grx 2 on the other hand (23.4 kDa) is atypical and has essentially no sequence identities apart from the active site compared to other Grx but it is highly active as a GSH oxidoreductase with 2-hydroxyethyl disulfide [8]. The activities of glutaredoxins from *E. coli* with protein-glutathione mixed disulfides are not known.

We have previously shown that there is a strong synergistic effect between PDI and *E. coli* Grx 1 during the early phase of glutathione-dependent folding of reduced RNase (RNase-SH) into the native and active protein [9]. Intermediates in the refolding of RNase-SH or glutathionylated RNase (RNase-SG) were characterized using mass spectrometry [10]. Grx 1 catalyzed both formation and reduction of mixed disulfides between RNase and GSH and in addition formation of native and non-native protein disulfide bonds. In comparison all protein disulfides generated in the presence of PDI were native.

This work focuses on the interaction of the three *E. coli* glutaredoxins and PDI with the fully glutathionylated model protein RNase-SG analyzing the kinetics of reduction of RNase-SG, and the effect of glutaredoxins in the refolding of RNase-SH and RNase-SG. We also suggest a mechanism for a possible ER-localized Grx.

2. Materials and methods

2.1. Materials

Glutaredoxins and PDI were prepared according to previously published procedures [5,11,12]. PD10 columns were purchased from Pharmacia Amersham Biotech, Filtron 3K concentrators were from Pall Filtron, Malmö, Sweden, Spectra/Por dialysis tubing was purchased from Labassco, Stockholm, Sweden. GSH, GSSG, RNase, DTT, NADPH and glutathione reductase were purchased from Sigma, Sweden. All other chemicals were analytical grade or better.

2.2. Determination of protein concentrations

Enzyme concentrations were determined from the absorbance at 280 nm using the following extinction coefficients: 12 500 M^{-1} cm^{-1} for Grx 1, 21 860 M^{-1} cm^{-1} for Grx 2, 4200 M^{-1} cm^{-1} for Grx 3, 47 300 M^{-1} cm^{-1} for PDI and 9800 M^{-1} cm^{-1} for RNase-SH and RNase-SG.

2.3. Preparation of RNase-SH and Rnase-SG

The RNase-SH was prepared as previously described [9]. To prepare RNase-SG, a solution of RNase-SH at about 4.0 mg/ml in 10 mM HCl was mixed with an equal volume of 8.0 M GuHCl in 0.1 M Tris, pH 8.0 and solid GSSG was dissolved to give a final concentration of 250 mM. The pH was adjusted to 8.0 with 10 M NaOH and the reaction between RNase-SH and GSSG was allowed to proceed at +4°C overnight. The bulk of GuHCl was removed by a brief dialysis against 0.1 M acetic acid using Spectra/Por dialysis tubing. Samples were further desalted by separation on PD10 columns in 0.1 M acetic acid and dialyzed four times against 1 l of 0.1 M acetic acid. Preparations of RNase-SG were then concentrated by centrifugation in Filtron 3K concentrators and analyzed for protein content (absorb-

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Abbreviations: DTT, dithiothreitol; ES-MS, electrospray mass spectrometry; Grx 1, 2 and 3, glutaredoxin 1, 2 and 3 from *Escherichia coli*; GS residue, glutathionyl residue; GuHCl, guanidinium hydrochloride; PDI, bovine protein disulfide isomerase; RNase, bovine pancreatic ribonuclease A type III; RNase-SG, ribonuclease A derivatized with glutathione; RNase-SH, fully reduced ribonuclease A

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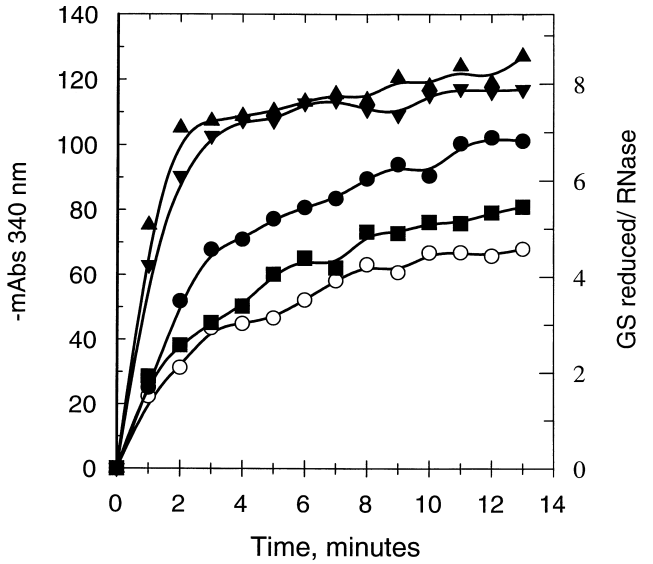

Fig. 1. Time course of the reduction of RNase-SG by Grx or PDI. ○ background, ▼ 50 nM Grx 1, ● 50 nM Grx 2, ▲ 50 nM Grx 3, ■ 500 nM PDI.

ance at 280 nm), thiol content [13], and free GSSG [14]. If the molar RNase:GSSG ratio was less than 2 the sample was desalted again and concentrated as before.

RNase-SG from three independent preparations was analyzed by electrospray mass spectrometry (ESMS). This showed that each preparation contained 45–55% of a species of 15 516 ± 3 Da, corresponding to RNase with six glutathionyl residues, and the rest was a species of 16 129 ± 3 Da, corresponding to RNase with eight glutathionyl residues. Such mixtures of RNase-SG containing six or eight glutathionyl residues were used in the refolding experiments.

RNase-SG containing eight glutathionyl groups was used to determine the specificity towards GS residues. This was prepared from mixtures of RNase-SG with six and eight glutathionyl residues by purification on a Sephasil C8 column on a SMART HPLC apparatus (Amersham Pharmacia Biotech). Two peaks were eluted with a linear gradient (16–28%) of acetonitrile in water and 0.1% TFA at a flow rate of 150 μl/min for 30 min. The first peak, which eluted at 22.5% acetonitrile, was pure RNase-SG with eight glutathionyl residues as confirmed by ESMS analysis. The derivatized protein was lyophilized and then dissolved in 10 mM HCl.

2.4. NADPH-dependent reduction of RNase-SG

Defined amounts of PDI or glutaredoxin were added to a cuvette containing 500 μl of 100 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM GSH, 100 μg/ml bovine serum albumin, 100 μM NADPH and yeast glutathione reductase 6 μg/ml and 2.4 μM RNase-SG. The absorbance was recorded at 340 nm against a control cuvette without RNase-SG, Grx or PDI. The initial rates were determined from the change in absorbance at 340 nm. For K_m and V_{max} determinations the rate of spontaneous reactions between GSH and RNase-SG was subtracted from the reaction rate in the presence of PDI of Grx. The values of the initial rates were plotted against the concentration of RNase-SG and the K_m and V_{max} were determined after fitting the data by non-linear regression to the equation $v = V_{max} \times [S]/(K_m + [S])$ using the program GraFit (Erithacus Software Ltd.).

2.5. Folding of RNase-SH and RNase-SG

Refolding RNase-SH or RNase-SG was measured by monitoring the increase of the rate of hydrolysis of 2′3′-cCMP which is a substrate for RNase, essentially as described [9,15,16]. Refolding reactions were carried out in the presence of 4.5 mM 2′3′-cCMP, 1 mM GSH, 0.2 mM GSSG in 80 mM Tris pH 8.0 and 1 mM EDTA. The concentration of RNase at each time point was calculated from the first derivative of the absorbance versus time data and corrected for depletion of cCMP and the product inhibition or RNase by CMP. The velocity (v) of RNase refolding in nM/min was determined from the slope of increase in RNase concentration and the lag phase was defined as the intercept of the straight line on the time axis (Fig. 2A). Rnase-SH and RNase-SG were always 8 μM.

3. Results and discussion

3.1. Reduction of mixed disulfides in RNase

It has previously been shown that in contrast to glutaredoxins, PDI does not catalyze the GSH-mediated reduction of the low molecular weight mixed disulfide between β-mercaptoethanol and GSH [9]. To investigate the specificity of glutaredoxins and PDI towards mixed disulfides of GSH and proteins, RNase was derivatized to contain eight glutathionyl residues per molecule. When this derivative was added to a reaction mixture containing NADPH, GSH and glutathione reductase, the observed oxidation of NADPH as a decrease in absorbance at 340 nm suggests that the following reactions occurred:

$$\mathrm{RNase-S-SG + GSH \xrightarrow{Grx} RNase-SH + GSSG} \quad (1)$$

$$\mathrm{GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+} \quad (2)$$

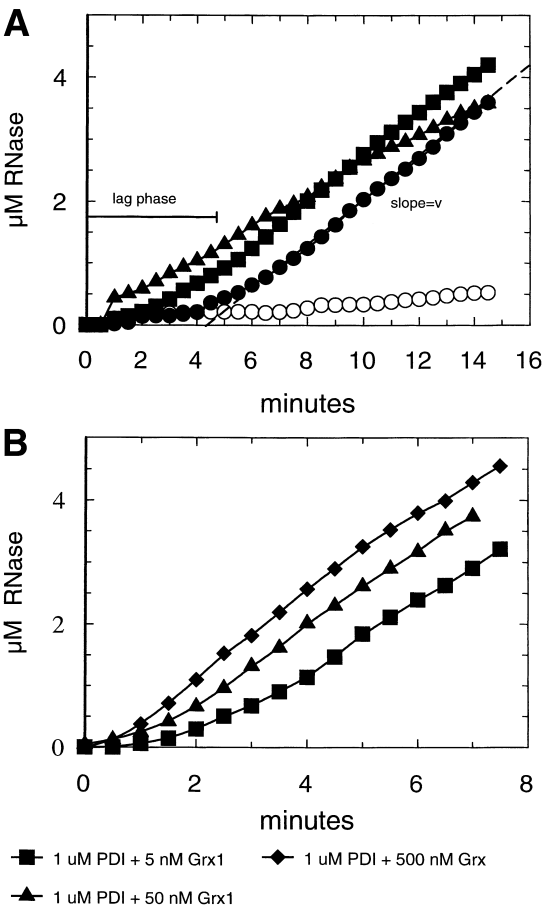

Fig. 2. Time course of refolding of RNase. Folding and disulfide bond formation in RNase. The reaction was started by addition of 8 μM RNase-SH in A and 8 μM RNase-SG in B. ○ background, ● 1 μM PDI+5 nM Grx 3, ■ 1 μM PDI+50 nM Grx 3, ▲ 1 μM PDI+500 nM Grx3.

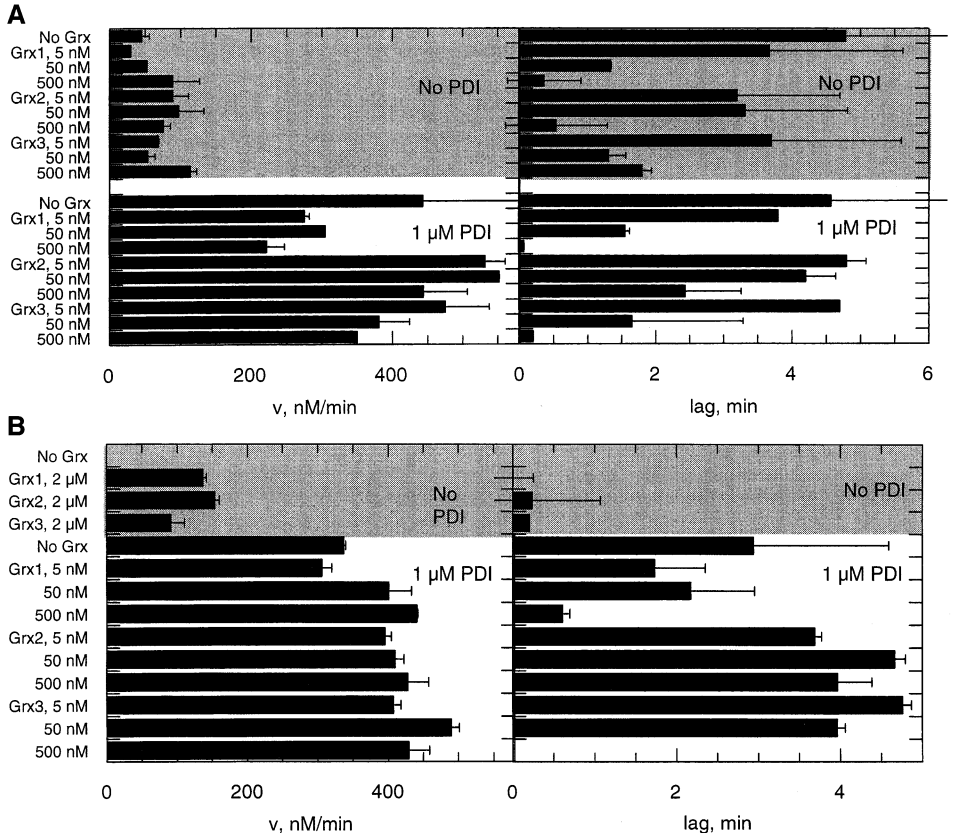

Fig. 3. Steady-state rates and lag times of refolding of RNase. Error bars represent standard errors. A: Refolding of RNase-SH. B: Refolding of RNase-SG.

$$\text{Net}: \text{RNase}-\text{S}-\text{SG}+\text{H}^{+}+$$

$$\text{NADPH}\rightarrow\text{RNase}-\text{SH}+\text{GSH}+\text{NADP}^{+} \quad (3)$$

Fig. 1 shows the time course of the reaction in the presence of 50 nM glutaredoxin or 500 nM PDI. All three Grxs were more efficient than PDI. At a concentration of 2.4 μM RNase-SG, corresponding to 19.2 μM GS residues, the reaction rate in the presence of 5 nM of Grx 1 or 3 was comparable to that catalyzed by 500 nM PDI (data not shown). In the presence of Grx 1 or 3 the reactions quickly approached completion in an apparently linear manner at a level exactly corresponding to eight molecules of NADPH oxidized for one molecule of RNase-SG, showing that all mixed disulfides were cleaved. The background reaction and the reaction catalyzed by PDI or Grx 2 appeared to occur in at least two phases but eventually reached the same level as that with Grx 1 or 3.

In a different experiment, the concentration of RNase-SG was varied up to 10 μM and the concentrations of Grx 1–3 and PDI were chosen so that the rate of the reactions was similar. The apparent K_m and k_{cat} numbers are shown in Table 1. Grx 1 and 3 had similar substrate saturation behaviors. Using Grx 2, we were unable to reach a substrate saturation level within the experimental limits and the reaction rate continued to increase in an apparently linear fashion at concentrations of 15–20 μM (data not shown). Grx 2 thus has a much higher apparent K_m (>25 μM) for GS residues and probably also a higher turnover number than Grx 1 and 3. Similar differences between Grx 2 and the other Grxs were obtained at pH 8.0 with 1 mM GSH and 0.7 mM hydroxyethyldisulfide [8]. The apparent K_m value of PDI was determined to be 5 μM and is thus similar to those of Grx 1 and 3. The large difference in catalytic activity resides entirely in a much lower turnover number of PDI compared to the three Grxs.

We have treated RNase-SG as one simple substrate and determined apparent K_m and k_{cat} values. This is an oversimplification since each GS residue may be differently accessible to the enzymes. Moreover, isomerization reactions will occur to liberate GSSG and form a protein disulfide bond within the RNase molecule. Such a disulfide bond might be the direct substrate for PDI. However, there is only one possible net reaction leading to reduced RNase, and it is clear that PDI is a much less efficient catalyst of it than the three Grxs.

3.2. *Oxidative folding of RNase-SH in the presence of PDI and Grx 1, 2 and 3*

Previously, we used PDI together with 1 or 0.5 μM concentrations of Grx 1 to catalyze oxidative folding of rd-RNase [9,10]. We have now examined the effect of different concentrations of Grx 1, 2 and 3 with PDI on the lag phase and steady-state rates. All three Grxs shortened the lag phase in a

Table 1
Apparent K_m and k_{cat} values of RNase-SG as a substrate

	K_m (μM)	k_{cat} (min^{-1})
Grx 1	2.2 ± 0.73	415 ± 66
Grx 2	>25	>150
Grx 3	2.3 ± 1.3	378 ± 29
PDI	5.0 ± 2.1	1.3 ± 0.4

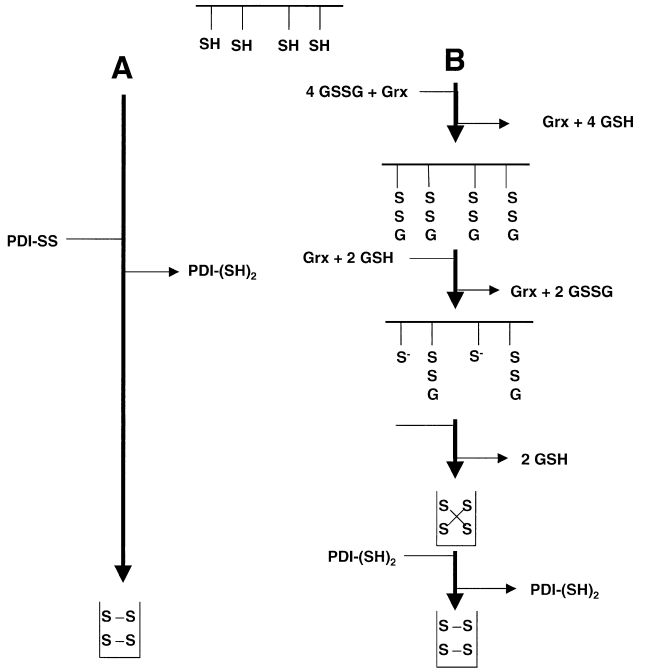

Fig. 4. Role and mechanism for a hypothetical Grx in the endoplasmic reticulum. See text for details.

concentration-dependent manner. Grx 1 and 3 are more efficient than Grx 2. In Fig. 2A, the time courses of the reactivation of RNase-SH in the presence of 1 μM PDI and different concentrations of Grx 3 are shown. As little as 5 nM of Grx 1 and 3 had a measurable effect and in the presence of 500 nM of Grx 1 or 3, we obtained apparently negative lag values. This has previously been attributed to an initial burst of enzymatic activity by fortuitous folding of a fraction of the substrate. A summary of the results of these experiments is shown in Fig. 3A. When the differences in activity in reduction of RNase-SG are considered, one might expect that Grx 2 may be applicable in vitro in refolding reactions with high protein concentrations.

The steady-state rate of RNase folding was subject to small variations within the experimental limits. This happened both in the presence and in the absence of PDI. However, systematic attempts to increase the concentrations of Grx 1–3 up to 10 μM did not result in any significant change in the steady-state rates. Other proportions of GSH to GSSG were examined to alter the redox status of the refolding buffer. None of the glutaredoxins had any influence on the steady-state rate but the lag phase was always shorter in the presence of Grxs.

3.3. Refolding and native disulfide bond formation in RNase-SG

RNase-SG containing six and eight glutathionyl residues at approximately equal amounts was used as a starting substrate in refolding reactions. Refolding of RNase-SG in the presence of PDI occurred with a similar lag time and at a similar rate as that of RNase-SH refolding. The time course of the reactions in the presence of Grx 1 is shown in Fig. 2B. No spontaneous reactivation of RNase occurred during the first 15 min. However, 2 μM Grx 1, 2 or 3 alone catalyzed refolding and activation of RNase-SG at a slow but measurable rate. Addition of 5, 50 or 500 nM of either of the three Grxs together with PDI did not yield any extra increase in the steady-state rates but the lag phase was shortened in a concentration-dependent manner by Grx 1 or 3. Grx 2 did not influence the reactions together with PDI (Fig. 3B).

To what extent a glutaredoxin activity in the ER lumen is required to assist in native disulfide formation is unknown. Our results indicate that PDI is unlikely to catalyze glutathione-dependent reactions if there is a Grx in the ER. In Fig. 4A a nascent polypeptide chain can be oxidized into the native form by PDI. This would require redox cycling of PDI probably via the recently identified gene product ERO1 [3]. Alternatively, non-native disulfide bonds could form by glutathione- and Grx-dependent oxidoreductions (Fig. 4B). At the redox conditions of the ER, the known Grxs catalyze both formation and reduction of GS protein mixed disulfides using the reactive N-terminal cysteine in a monothiol reaction mechanism. The non-native protein disulfides then require isomerization by reduced PDI, but no further redox cycling of PDI would be necessary.

An example of a non-cytosolic type of glutaredoxin is encoded by the protozoan parasite *Thelia parva* and implicated in the host bovine T-cell blast transformation. This is the first example of a glutaredoxin that enters the secretory pathway in eukaryotic cells [17].

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