FEBS 16484 FEBS Letters 378 (1996) 69–73

High-level expression of fully active human glutaredoxin (thioltransferase) in *E. coli* and characterization of Cys^7 to Ser mutant protein

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Received 20 November 1995

Abstract **Glutaredoxin (Grx) (12 kDa) is a hydrogen donor for ribonucleotide reductase and also a general GSH-disulfide reductase of importance for redox regulation. To overexpress human glutaredoxin in *Escherichia coli*, a cDNA encoding human Grx was modified and cloned into the vector pET-3d and expressed in *E. coli* BL21(DE3) by IPTG induction. High-level expression of Grx was verified by GSH-disulfide oxidoreductase activity, SDS-PAGE and immunoblotting analysis. The recombinant human Grx in its reduced form was purified to homogenity with 50% yield and exhibited the same dehydroascorbate reductase and hydrogen donor activity for ribonucleotide reductase (K_m ~ 0.2 μM) as the human placenta protein. Human Grx contains a total of 5 half-cystine residues including a non-conserved Cys^7 residue and is easily oxidized to form dimers during storage. A Grx mutant Cys^7 to Ser was generated by site-directed mutagenesis and the protein was purified to homogeneity. The mutant protein showed full activity and exhibited a much reduced tendency to form dimers compared with the wild type protein. Peptide sequencing confirmed the mutation and removal of the N-terminal Met residue in both wild type and mutant proteins. Fluorescence spectra demonstrated only tyrosine fluorescence in human Grx with a peak at 310 nm which increased 20% upon reduction and decreased by addition of GSSG demonstrating that glutathione-containing disulfides are excellent substrates.**

Key words: Glutaredoxin; Thioltransferase; Thiol-disulfide; Recombinant protein; Site-directed mutagenesis; Fluorescence

1. Introduction

Glutaredoxin (Grx) is a small protein (M_r = 12,000) containing the active site -Cys-Pro-Tyr-Cys- which is conserved in such different organisms as *E. coli*, vaccinia virus, yeast, plants and mammalian cells [1–8]. Grx was originally discovered in *E. coli* and calf thymus as a GSH-dependent hydrogen donor for ribonucleotide reductase [9–12], an essential enzyme for DNA synthesis. Glutaredoxin has inherent glutathione-disulfide oxidoreductase activity in a coupled system with GSH, NADPH and glutathione reductase [12], catalyzing the reduction of low molecular weight disulfides as well as proteins. Grx has been proposed to exert a general thiol redox control of protein activity by acting both as an effective protein disulfide reductase, similar to thioredoxin [13,14], and as a specific GSH-mixed disulfide reductase.

Mammalian glutaredoxins from calf thymus, rabbit bone marrow, pig liver and human placenta contain two structural half-cystine residues in the C-terminal part of potential regulatory role since their oxidation inactivates enzyme activity [5]. Those two cysteines are part of a group of amino acids conserved among the TGF-β family and animal glutaredoxins [15]. Glutaredoxin also exhibits dehydroascorbate reductase activity, suggesting a function to defend cells against oxidative stress [16].

Glutaredoxin genes (*grx*) have been cloned from *E. coli*, yeast and pig [17–21]. Grx from placenta was previously purified to homogeneity (12 kDa) and its amino acid sequence was determined (105 residues) by peptide sequencing and a cDNA encoding the protein was cloned and sequenced [8]. Human Grx contains one extra Cys residue (Cys^7) apart form the two active site cysteines and the previously mentioned two conserved half-cystine residues; the human protein therefore particularly easy forms multimers by disulfide bridges upon oxidation.

In this article we describe the high-level expression of human glutaredoxin in *E. coli* and purification and characterization of recombinant wild type and a Cys^7 to Ser mutant protein.

2. Materials and methods

2.1. Materials

Purified recombinant mouse ribonucleotide reductase was a kind gift of Prof. L. Thelander of the University of Umeå (Sweden). Antibodies against human placenta glutaredoxin were generated in a goat (Padilla et al. to be published). All other chemicals and reagents were of analytical grade.

2.2. Methods

2.2.1. Construction of human glutaredoxin expression vector. The entire cDNA encoding human glutaredoxin [8] was amplified by PCR from plasmid pGEM(*grx*) by using two mutagenic primers (5′-CATCGCCATGGCTCAAGA-3′ and 5′-TTCCCATGGGATCTGTGGT-3′; the underlined bases were the mutations relative to the original cDNA sequence) to introduce a *Nco*I site at the initiation codon region and a *Nco*I site after the stop codon region (bold letters). The amplified DNA was cloned in expression vector pET-3d [22] and *E. coli* strain BL21(DE3) was transformed with pET-*grx*.

2.2.2. Construction of a mutant (Cys^7 to Ser) expression vector. The procedure was the same that we have described above but changing the mutagenic primer at the 5′ end. The new primer (5′-ATCGCCATGGCTCAAGAGTTTGTGAACA GCA-3′) has a mutation to introduce a *Nco*I site at its initiation codon region (underlined base) and another one to change Cys^7 to Ser (double underlined base).

2.2.3. Expression and purification of recombinant human glutaredoxin. Transformed *E. coli* BL21(DE3) cells with PET-*grx*

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Abbreviations: BSA, bovine serum albumin; DTT, dithiolthreitol; GSH, glutathione; GSSG, oxidized glutathione; HED, β-hydroxyethylene disulfide; PMSF, phenylmethylsulfonyl fluoride; DHA, dehydro L-ascorbate; IPTG, isopropyl β-D-thiogalactopyranoside; NMR, nuclear magnetic resonance; TGF, transforming growth factor.

The gene sequence reported in this paper has been deposited in the GenBank data base (accession no. X76648).

SSDI 0014-5793(95)01413-6

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                                                        CATCGCC    7
ATG GCT CAA GAG TTT GTG AAC TGC AAA ATC CAG CCT GGG AAG GTG   52
Met Ala Gln Glu Phe Val Asn Cys Lys Ile Gln Pro Gly Lys Val   15

GTT GTG TTC ATC AAG CCC ACC TGC CCG TAC TGC AGG AGG GCC CAA   97
Val Val Phe Ile Lys Pro Thr Cys Pro Tyr Cys Arg Arg Ala Gln   30

GAG ATC CTC AGT CAA TTG CCC ATC AAA CAA GGG CTT CTG GAA TTT  142
Glu Ile Leu Ser Gln Leu Pro Ile Lys Gln Gly Leu Leu Glu Phe   45

GTC GAT ATC ACA GCC ACC AAC CAC ACT AAC GAG ATT CAA GAT TAT  187
Val Asp Ile Thr Ala Thr Asn His Thr Asn Glu Ile Gln Asp Tyr   60

TTG CAA CAG CTC ACG GGA GCA AGA ACG GTG CCT CGA GTC TTT ATT  232
Leu Gln Gln Leu Thr Gly Ala Arg Thr Val Pro Arg Val Phe Ile   75

GGT AAA GAT TGT ATA GGC GGA TGC AGT GAT CTA GTC TCT TTG CAA  277
Gly Lys Asp Cys Ile Gly Gly Cys Ser Asp Leu Val Ser Leu Gln   90

CAG AGT GGG GAA CTG CTG ACG CGG CTA AAG CAG ATT GGA GCT CTG  322
Gln Ser Gly Glu Leu Leu Thr Arg Leu Lys Gln Ile Gly Ala Leu  105

CAG TAA CCACAGATCCCATGGGAA                                  346
Gln ***                                                     106
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Fig. 1. Complete nucleotide sequence and deduced amino acid sequence of the human glutaredoxin cDNA modified for the overexpression of the protein *E. coli*. The initiator Met and the two *Nco*I sites are bold type. The translation termination signal is indicated by (***). The internal primers used for sequencing are underlined.

were cultured at 37°C in 4 l of fresh Luria-Bertani medium with 50 μg/ml of ampicillin and shaken until an A_{550} of 0.5 was obtained. Then, IPTG was added to a final concentration of 0.5 mM and the culture was continuously shaken at 37°C for 3.5 h. The following steps were carried out at 4°C. Cells were harvested by centrifugation at 10,000 × *g* for 20 min and the pellet (10 g) was resuspended in 50 ml of 50 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM PMSF. Lysozyme was added to a final concentration of 0.2 mg/ml with stirring for at least 30 min and the cells were disrupted by sonication for 20 min (60 s × 30 s intervals). Insoluble material was sedimented at 15,000 × *g* for 30 min. After centrifugation the supernatant fraction was placed in a fresh tube and streptomycin sulphate was added to 0.5% saturation to precipitate nucleic acid. The mixture was stirred for 1 h. The suspension was centrifuged for 30 min at 15,000 × *g* and the supernatant was fractionated by using ammonium sulphate 40% and 85% saturation to precipitate protein. The pellet from 85% saturation was dissolved in 30 ml of buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1 mM DTT), and dialyzed against the same buffer. This preparation was preincubated with 2 mM DTT at 37°C for 15 min and loaded onto a DEAE-Sephacel column (12 × 3.5 cm) that had been equilibrated with buffer A. The recombinant Grx did not bind under those conditions. The flow-through fractions containing activity were concentrated by ammonium sulphate precipitation (85%) and the pellet was resuspended in 3.5 ml of buffer B (10 mM sodium phosphate buffer pH 6.8, 0.1 mM EDTA). The sample was preincubated with 2 mM DTT at 37°C for 15 min and applied to a Sephadex G-50 column (142 × 1.5 cm), equilibrated with buffer B. The active fractions were pooled (80 ml), concentrated by Diaflo ultrafiltration to 20 ml, incubated with 10 mM HED at room temperature for 30 min and loaded onto a CM-Sepharose column (17 × 1.6 cm) equilibrated with buffer B. The recombinant human glutaredoxin was eluted with a 300 ml total volume linear gradient of 0.0–0.5 M NaCl in buffer B. The protein concentration was determined as described by Bradford [23] using a BSA standard curve.

Expression and purification of recombinant human glutaredoxin mutant were the same that we have described previously for the wild type protein.

2.2.4. Assay of enzymatic activities. Glutathione-disulfide oxidoreductase activity was determined as described by Luthman and Holmgren with HED [12] or dehydroascorbate [16] as substrates. The activity of the recombinant human glutaredoxin with the heterologous mouse ribonucleotide reductase was determined as described by Luthman and Holmgren [12]. The R2 subunit of ribonucleotide reductase was reactivated following the anaerobic procedure described by Mann et al. [24].

2.2.5. SDS-PAGE, immunoblotting analysis and isoelectric focusing. SDS-PAGE was performed on Pharmacia Phast System using phast gel homogeneous 20%. The separated proteins were electrophoretically transferred to nitrocellulose membranes (0.45 μm) and the blots were developed as described by Rozell et al., [25] using affinity-purified goat anti-human glutaredoxin as primary antibodies. Isoelectric focusing was performed on a Pharmacia Phast System using gels 3-10 supplied by the manufacturer and silver staining.

2.2.6. Fluorescence measurements. Protein fluorescence was measured with a thermostated SPEX-FluoroMax spectrofluorometer at

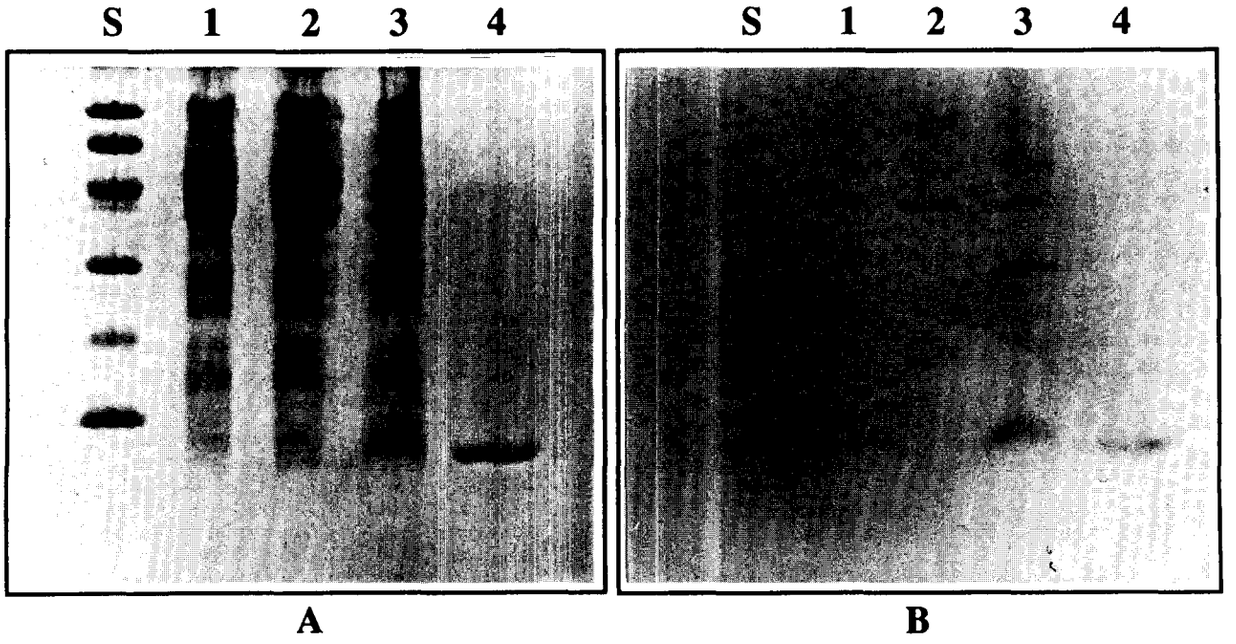

Fig. 2. Expression of the human glutaredoxin in *E. coli*. Total bacterial lysate proteins were resolved by SDS/PAGE and stained with Coomassie blue (panel A) or analyzed by immunoblotting (panel B). Lane S, Pharmacia low molecular weight protein standards (14000–94000). Lane 1, control *E. coli* BL21(DE3). Lane 2, cells transformed with the pET-3d vector and induced with IPTG. Lane 3, cells transformed with the pET-*grx* vector and induced with IPTG. Lane 4, purified native human glutaredoxin (0.5 μg).

15°C. Excitation of fluorescence was at 230 nm and emission spectra from 250 to 400 nm were recorded. Emission at 310 nm was used to follow oxido-reductions. A preparation of 66 μM recombinant human Grx was reduced by incubation with 1 mM DTT for 15 min at 37°C, or oxidized by addition of 100 μM GSSG. The samples for fluorescence measurements contained 3.3 μM concentration of human glutaredoxin in 2 ml of N_2-equilibrated 0.1 M potassium phosphate pH 7.0, 1 mM EDTA buffer. Denaturation of glutaredoxin was achieved by incubation in the above buffer with 6 M guanidine hydrochloride.

3. Results and discussion

3.1. Construction of human glutaredoxin expression vector

Two *Nco*I sites were introduced by PCR, using mutagenic primers (see section 2) in the cDNA encoding human glutaredoxin [8]: one at the translation initiation codon region and the other after the stop codon. The amplified DNA, which had the expected size (343 bp), was digested with *Nco*I, gel-purified and cloned in the expression vector pET-3d under control of a T7 promotor.

E. coli BL21(DE3) competent cells were transformed with the constructed expression vector pET-*grx* and purified plasmid DNA was sequenced using T7 and T3 promotor primers and two internal primers. The full nucleotide sequence of the insert was obtained (343 bp). The insert was in the right orientation for expression and its sequence was in complete agreement with the cDNA sequence encoding human glutaredoxin, except for the two new *Nco*I sites that we introduced for direct cloning in pET-3d vector (Fig. 1).

3.2. Expression of recombinant human glutaredoxin

The expression of recombinant human glutaredoxin in transformed bacterial culture, was induced by the addition of IPTG for 3.5 h. After induction, an aliquot of the bacterial culture (0.5 ml) was centrifuged, resuspended in loading buffer and total cellular proteins were separated by SDS-PAGE (Phast gel homogeneous 20) and stained with Coomassie blue. Induction with IPTG resulted in the appearance of a prominent protein which migrated at the same position as purified glutaredoxin from human placenta (12 kDa); it was not present in *E. coli* BL21(DE3) or transformed bacteria with the control plasmid pET-3d and induced with IPTG (Fig. 2A). The overexpression of recombinant human Grx was also verified by immunoblotting analysis using affinity-purified goat antibodies against glutaredoxin from human placenta (Fig. 2B).

Table 1
Purification of recombinant human glutaredoxin from 10 g of *E. coli* BL21(DE3)/PET/grx cells

Steps	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Extract	600	23,870	39	100
Ammonium sulfate precipitation	468	19,750	42	83
DEAE-Sephacel	115	14,375	125	60
G-50 Sephadex	48	13,246	276	55
CM-Sepharose	30	12,000	400 (29)* (0.7)**	50

*DHA reductase activity.
**Ribonucleotide reductase activity (nmol dCDP/30 min).

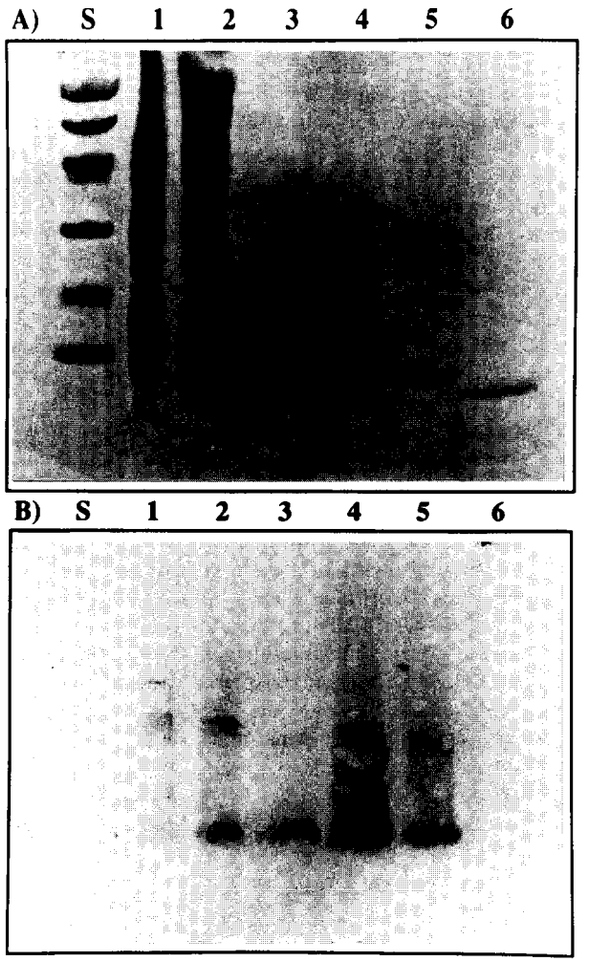

Fig. 3. Purification of recombinant human glutaredoxin. Samples of protein from the different steps were resolved by SDS-PAGE and stained with Coomassie blue (panel A) or analyzed by immunoblotting (panel B). Lane S, Pharmacia low molecular weight protein standards (14,000–94,000). Lane 1, 10 μg of cell extract. Lane 2, 10 μg of ammonium sulfate precipitate. Lane 3, 1 μg of DEAE-Sephacel chromatography pool. Lane 4, 1μg of G-50 Sephadex chromatography pool. Lane 5, 0.5 μg of CM-Sepharose chromatography pool. Lane 6, 0.5 μg of *E. coli* glutaredoxin.

3.3. Purification and characterization of recombinant human glutaredoxin

The recombinant glutaredoxin was purified to homogeneity by ammonium sulphate precipitation, cation-exchange, gel filtration and anion exchange column chromatography steps as described in section 2. The procedure was similar to that used in the purification of glutaredoxin from human placenta [8] but we excluded the first CM-Sepharose chromatography for reduced Grx, using only the second one for oxidized Grx.

Samples from different steps in the purification were analyzed by SDS-PAGE (phast gel homogeneous 20) and immunoblotting (Fig. 3A and 3B). A single band of 12 kDa was detected after the CM-Sepharose chromatography, showing strong reaction with antibodies against human glutaredoxin. These antibodies did not cross-react with either *E. coli* Grx1 (Fig. 3) or Grx2 or Grx3 (data not shown).

The results of the purification of the recombinant human

glutaredoxin are summerized in Table 1. The purified protein (30 mg) was obtained in 50% yield and has a specific activity of 400 U/mg with HED and 29 U/mg with DHA. It also showed activity as hydrogen donor for heterologous mouse ribonucleotide reductase comparable to that of the protein purified from human placenta [8].

The three different assayed activities with the recombinant glutaredoxin gave the same values as those of glutaredoxin from human placenta [8]. Thus, the expression system used successfully produced large amounts of fully active human glutaredoxin. Peptide sequencing showed absence of Met residue at the N-terminal (data not shown).

Isoelectric focusing analysis of the native and recombinant human glutaredoxin revealed that the pI values for the oxidized and reduced forms are the same in both proteins: 9.0 in its HED-oxidized state and 7.3 in its DTT-reduced form (Fig. 4).

We have analyzed the fluorescence of human glutaredoxin in their native oxidized and reduced states as well as in their denatured forms using 6 M guanidine-HCl. Fluorescence emission spectra of human Grx, adjusting the excitation wavelength to 230 nm, showed a peak at 310 nm but not at 350 nm (data not shown). This is consistent with the amino acid composition of the protein [8] which has two tyrosine residues (one at the active site) but no tryptophan residue which gives fluorescence at 350 nm. Fully reduced human glutaredoxin had higher fluorescence emission than that of the GSSG-oxidized form and this difference between non-denatured oxidized and reduced

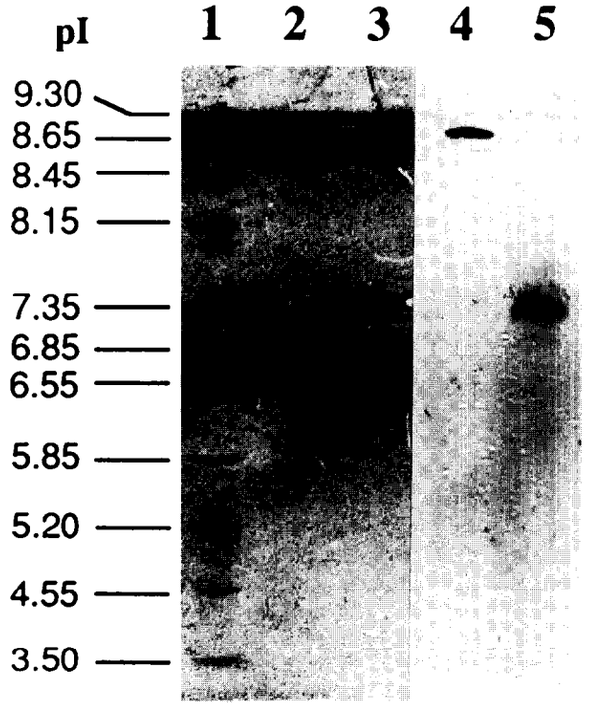

Fig. 4. Isoelectric focusing of human glutaredoxin. Purified recombinant and native proteins were subjected to IEF in the range of pH 3–10 and silver stained. Lane1, Pharmacia pI standards. Lane 2, 0.25 μg of native human glutaredoxin treated with 2 mM DTT for 15 min at 37°C. Lane 3, 0.25 μg of reduced native human glutaredoxin treated with 10 mM HED for 30 min at room temperature. Lane 4, 0.25 μg of recombinant human glutaredoxin treated with 2 mM DTT for 15 min at 37°C. Lane 5, 0.25 μg of reduced recombinant human glutaredoxin treated with 10 mM HED for 30 min at room temperature.

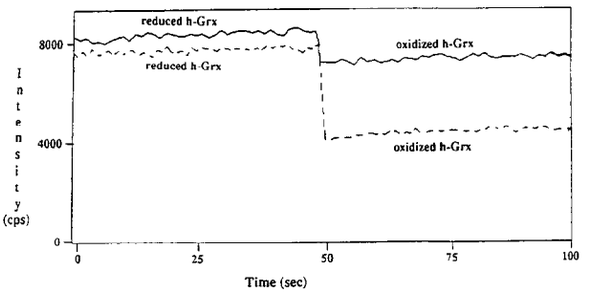

Fig. 5. Relative tyrosine fluorescence of oxidized and reduced human glutaredoxin (3.3 μM). To the reduced form, 100 μM GSSG was added after 50 s. Solide line corresponds to non denatured protein and dashed line to denatured protein. The emission wavelength was 310 nm.

states was higher in the denatured protein (Fig. 5). In human Grx, this effect was observed at 230 nm of excitation, suggesting disulfide quenching of the tyrosine residue at the active site ($Tysr^{24}$) in oxidized glutaredoxin by mechanisms similar to that for tryptophan fluorescence in *E. coli* thioredoxin [26] or calf thymus protein disulfide isomerase [27]. The structure of *E. coli* Grx has been determined [28–30] and the active site disulfide is located in a loop after the first β-strand (β1) and is followed by a long α-helix (α1). The structure of human Grx is unknown but a similar localization of the active site was predicted [8].

3.4. *Expression, purification and characterization of recombinant human glutaredoxin mutant (Cys⁷ to Ser)*

The procedure was the same as described for expression of human Grx wild type but changing the mutagenic primer at the 5′ end. The full nucleotide sequence of the insert was obtained and the mutation at the right position was confirmed. The rest of the sequence was exactly the same as that obtained for the insert of the human Grx expression vector. The purification procedure was identical to that described for the purification of recombinant wild type Grx and peptide sequencing confirmed the Ser residue and no N-terminal Met-residue (data not shown). The pure mutant protein had the same properties and specific activity as the wild type protein but showed much lower tendency to form dimers. If this extra Cys residue, not present in other mammalian glutaredoxins, plays a special role in human glutaredoxin,it will be subject to further studies.

The expression system described in this paper will be usefull for production of recombinant protein to investigate the molecular properties of glutaredoxin by multidimensional NMR or X-ray crystallography and site-directed mutagenesis.

Acknowledgements: We are grateful to Prof. L. Thelander of the University of Umeå (Sweden) for providing the purified recombinant mouse ribonucleotide reductase. We thank Åsa Michelgård for her help in the molecular biology techniques and Frederik Åslund in peptide sequencing. This research was supported by grants form the Swedish Cancer Society (961), Swedish Medical Research Council (Project 13X-10370 and 13P-10636), the Lundberg Foundation, the K.A. Wallenberg Foundation and the Karolinska Institute. Dr. C.A. Padilla was supported by a fellowship from the European Communities.

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