



## Review

## Glutaredoxin systems

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## ARTICLE INFO

## Article history:

Received 19 May 2008

Received in revised form 11 June 2008

Accepted 11 June 2008

Available online 18 June 2008

## Keywords:

Glutaredoxin

Thioredoxin

Glutathione

Redox control

Redox signaling

Iron–sulfur cluster

Iron homeostasis

## ABSTRACT

Glutaredoxins utilize the reducing power of glutathione to maintain and regulate the cellular redox state and redox-dependent signaling pathways, for instance, by catalyzing reversible protein S-glutathionylation. Due to the general importance of these processes, glutaredoxins have been implied in various physiological and disease-related conditions, such as immune defense, cardiac hypertrophy, hypoxia-reoxygenation insult, neurodegeneration and cancer development, progression as well as treatment. The past years have seen an impressive gain of knowledge regarding new glutaredoxin systems and functions. This is true both with respect to new functions in redox regulation and also with respect to unexpected new ties to iron metabolism and iron–sulfur cluster biosynthesis. The aim of this review is to provide a state-of-the-art overview over these recent discoveries with a focus on aspects related to human health.

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## 1. Introduction

Glutaredoxins (Grxs) have been first described three decades ago as glutathione-dependent reductases (*redoxins*) of the disulfide formed in ribonucleotide reductase during its catalytic cycle, when Grx was able to restore the growth of *Escherichia coli* in a mutant lacking thioredoxin (Trx) [1–3]. Trxs and Grxs share a number of additional functions, however, it soon became obvious that Grxs, compared to Trxs, are more versatile with respect to the choice of substrate and reaction mechanisms. Moreover, in addition to the early discovered dithiol Grxs containing the characteristic Cys-Pro-Tyr-Cys active site motif, sequence information from various genomic projects and functional studies during the last few years revealed a second group of Grxs. This group, commonly named monothiol Grxs, lacks the C-terminal active site thiol in its Cys-Gly-Phe-Ser active site but contains all structural and functional elements to bind and utilize GSH as substrate. Based on these discoveries and the increasingly recognized importance of redox control for cellular function, the Grx

field is still strongly progressing and expanding. At the time of writing Pubmed lists approx. 800 entries for “glutaredoxin” from which a little less than half date back to the last 5 years. This aim of this review is to provide an overview over these recent developments, especially in relation to human health. Of course, many of these aspects have to be discussed in perspective of the ground-breaking work stemming from bacteria, fungi, plants and other model organisms.

## 1.1. The glutathione redox couple

The tripeptide glutathione ( $\gamma$ -glutamyl-cysteinyl-glycine, GSH) is the major biological thiol compound and plays a pivotal role as buffer of the cellular redox state and in antioxidant defense [4]. It is present in millimolar concentrations in the cell and the major determinant of the cellular redox state. The glutathione redox couple GSH/glutathione disulfide (GSSG) can transfer two electrons (Eq. (1)). Most organisms reduce GSSG with the help of the dimeric flavoenzyme glutathione reductase (GR) at the expense of NADPH (Eq. (2)).

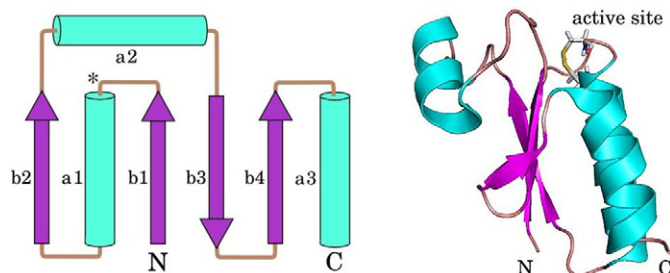


The commonly used [GSH] to [GSSG] ratio does not reflect the cellular redox state very well, because this ratio does not take into account the stoichiometry of the reaction and neglects the potentiating effect of GSH depletion (see also ref. [5]). Instead, approximations of the cellular redox state should be based on the [GSH]<sup>2</sup> to [GSSG] ratio. The standard redox potential  $E^0$  for the GSH/GSSG redox couple

*Abbreviations:* AD, Alzheimer's disease; AFT, activator of ferrous transport; APS, adenylylsulfate; ASK1, apoptosis signaling kinase 1; COPD, chronic obstructive pulmonary disease; DTT, dithiothreitol; ET-1, endothelin-1; Fur, ferric uptake regulator; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulfide; HED, hydroxyethyl disulfide; IRE, iron regulatory element; IRP, iron regulatory protein; MLP, muscle LIM protein; NFAT, nuclear factor of activated T cells; PAPS, phospho adenylylsulfate; PD, Parkinson's disease; PE, phenylephrine; RNR, ribonucleotide reductase; TGR, thioredoxin glutathione reductase; Trx, thioredoxin;  $\beta$ -ME,  $\beta$ -mercaptoethanol

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**Fig. 1.** Glutaredoxin structure. Bacterial glutaredoxins exhibit the most basic representation of the thioredoxin fold (left site). The structure of oxidized *E. coli* Grx1 is shown (PDB accession number 1EGO).

is  $-240$  mV. Hence, the cellular redox state can be approximated according to the Nernst equation as shown in Eq. (3).

$$E = -240[\text{mV}] + (R \cdot T / 2 \cdot F) \cdot \ln\left(\frac{\text{GSSG}}{\text{GSH}^2}\right) \quad (3)$$

The cellular (GSH) redox state changes in response to external stimuli and in response to the state of the cell. For instance, proliferation occurs at approximately  $-240$  mV, differentiation at approximately  $-200$  mV, and apoptosis at approximately  $-170$  mV [6,5]. The reaction rates of GSH and GSSG with protein thiols are normally too slow to be of importance under physiological conditions, however, the values of the cellular GSH-GSSG redox potential are close to the midpoint potential for Grxs, that can be GSH-dependent reductases at  $-240$  mV, or GSSG-dependent oxidases at  $-170$  mV [7]. Grxs are therefore ideal candidates for the regulation of cellular processes associated with changes in the GSH-GSSG redox state.

## 1.2. Structure of glutaredoxins

Grxs have been studied intensively by both X-ray crystallography and NMR spectroscopy. At present, around 40 structures of dithiol and one structure of a monothiol Grx are available in the protein database. Structurally, Grxs belong to the Trx fold family of proteins. In fact, bacterial Grxs display the most basic representation of the Trx fold, while it represents only a substructure or a domain in the other members of the family [8,9] (Fig. 1). This motif consists of a four stranded  $\beta$ -sheet surrounded by three  $\alpha$ -helices (Fig. 1). In addition, all oxidoreductases of the Trx family of proteins share a similar active site motif (Cys-X-X-Cys or Cys-X-X-Ser) located on the loop connecting  $\beta$ -sheet 1 and  $\alpha$ -helix 1 (Fig. 1\*) and a *cis*-Pro residue. The N-terminal Cys residue in the active site of Grxs is, similar to Trxs, surface exposed and has a low pKa value, i.e. 3 or more pH units below the pKa of free Cys, while the more C-terminal Cys is buried in the molecule and has a much higher pKa value.

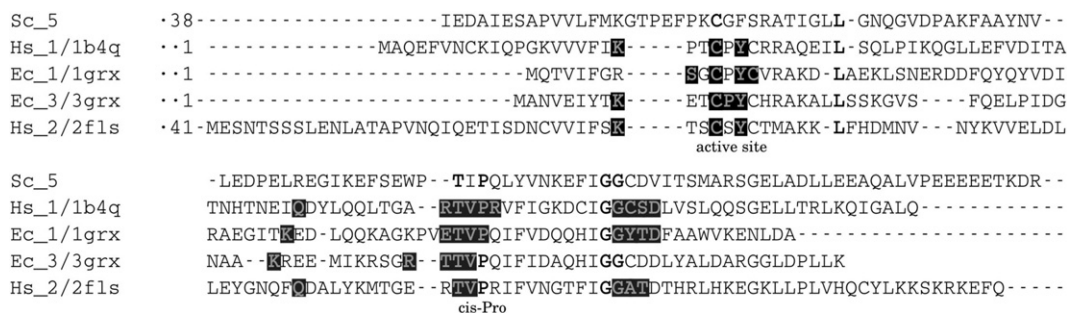
Grxs were defined by their ability to bind and utilize GSH as substrate. The structures of GSH-mixed disulfide intermediates (see below) and the recent structures of [Fe-S] Grxs with-covalently bound GSH have provided

valuable insights into the GSH binding sites (Fig. 2). Next to the active site residues, two additional areas step out: the residues preceding the *cis*-Proline (consensus: TVP) and the residues following the Grx-characteristic GG-motif (consensus: GGxdD). In addition, two more positively charged residues N-terminal of the active site and the TVP motif take part in aligning the substrate GSH. Utilizing these motifs, Grxs bind the GSH moiety in at least three distinct modes (Figs. 2 and 3). First, in a mixed disulfide intermediate with the N-terminal active site thiol following a nucleophilic attack on a GSH-mixed disulfide substrate. Secondly, this mixed disulfide can be attacked by a second molecule of GSH to release the mixed disulfide intermediate. Thirdly, the subgroup of [Fe-S]-binding Grxs can bind GSH non-covalently with the thiol group of both the N-terminal active site and the GSH thiol coordinating the metal cofactor.

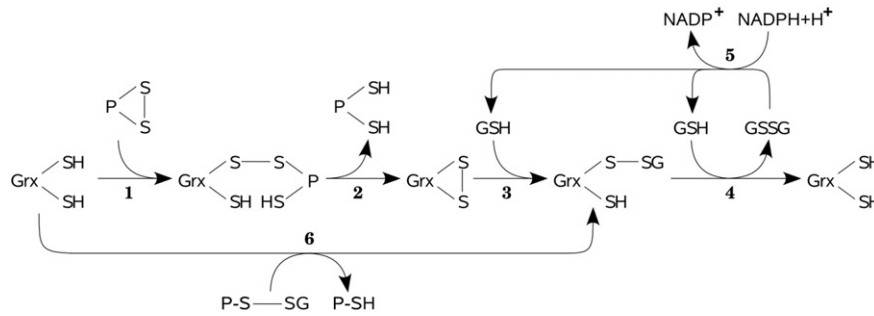
## 1.3. Reaction mechanisms

Grxs are versatile oxidoreductases able to reduce a variety of substrates including at least one compound devoid of thiol groups. Two distinct but functionally connected reaction mechanisms evolved, the dithiol and the monothiol mechanism, that both rely on the proteins' inherent affinity for the GSH moiety [10–16]. Similar to Trxs, a number of Grxs catalyse the reversible reduction of protein disulfides utilizing both cysteinyl residues in their Cys-Pro-Tyr-Cys active site (Fig. 3). In the first step, the more N-terminal Cys residue performs a nucleophilic attack on the target disulfide. Next, the mixed disulfide intermediate formed between the two proteins is attacked by the second active site thiolate. The resulting disulfide in the active site is reduced by one molecule of GSH leading to a mixed disulfide between GSH and the N-terminal active site cysteine. This mixed disulfide is subsequently reduced by a second GSH molecule. The reduction of disulfides formed between glutathione and proteins or small molecular weight compounds requires only the N-terminally located active site Cys residue. In this reaction, Grxs show a clear preference for the non-GSH molecule as leaving group, whereas GSH forms a mixed disulfide with the N-terminal thiol. As described for the dithiol mechanism, this disulfide is reduced by the second molecule of GSH. The resulting glutathione disulfide (GSSG) is regenerated by glutathione reductase at the expense of NADPH.

Ascorbic acid is an important antioxidant and essential for the activity of hydroxylases of the collagen synthesis pathway. Oxidation of ascorbate with two electrons yields dehydroascorbate. Ascorbate can be regenerated by a number of oxidoreductases including Grxs, protein disulfide isomerase, but not Trxs [17]. Based on biochemical studies two reaction mechanisms were proposed for the GSH-dependent reduction of dehydroascorbate by Grxs [18] similar to the monothiol and dithiol reaction mechanisms. In this model the N-terminal active site thiolate attacks carbon 2 of the dehydroascorbate molecule. The intermediate thiohemiketal is subsequently reduced by the C-terminal thiol or one molecule of GSH leading to the active site disulfide or a mixed disulfide of the N-terminal thiol with GSH that are further reduced as described above.



**Fig. 2.** Residues involved in GSH binding in different Grxs. The residues in direct molecular contact with the GSH molecule are shaded black and green. Molecular contacts were analyzed using Whatif [199]. The PDB accession numbers of the structures analyzed are indicated, i.e. the mixed disulfides of GSH with mutants of human Grx1 and *E. coli* Grx1 and 3 and the non-covalent complex between apo-Grx2 and GSH. Yeast Grx5 was used for comparison as model monothiol Grx.



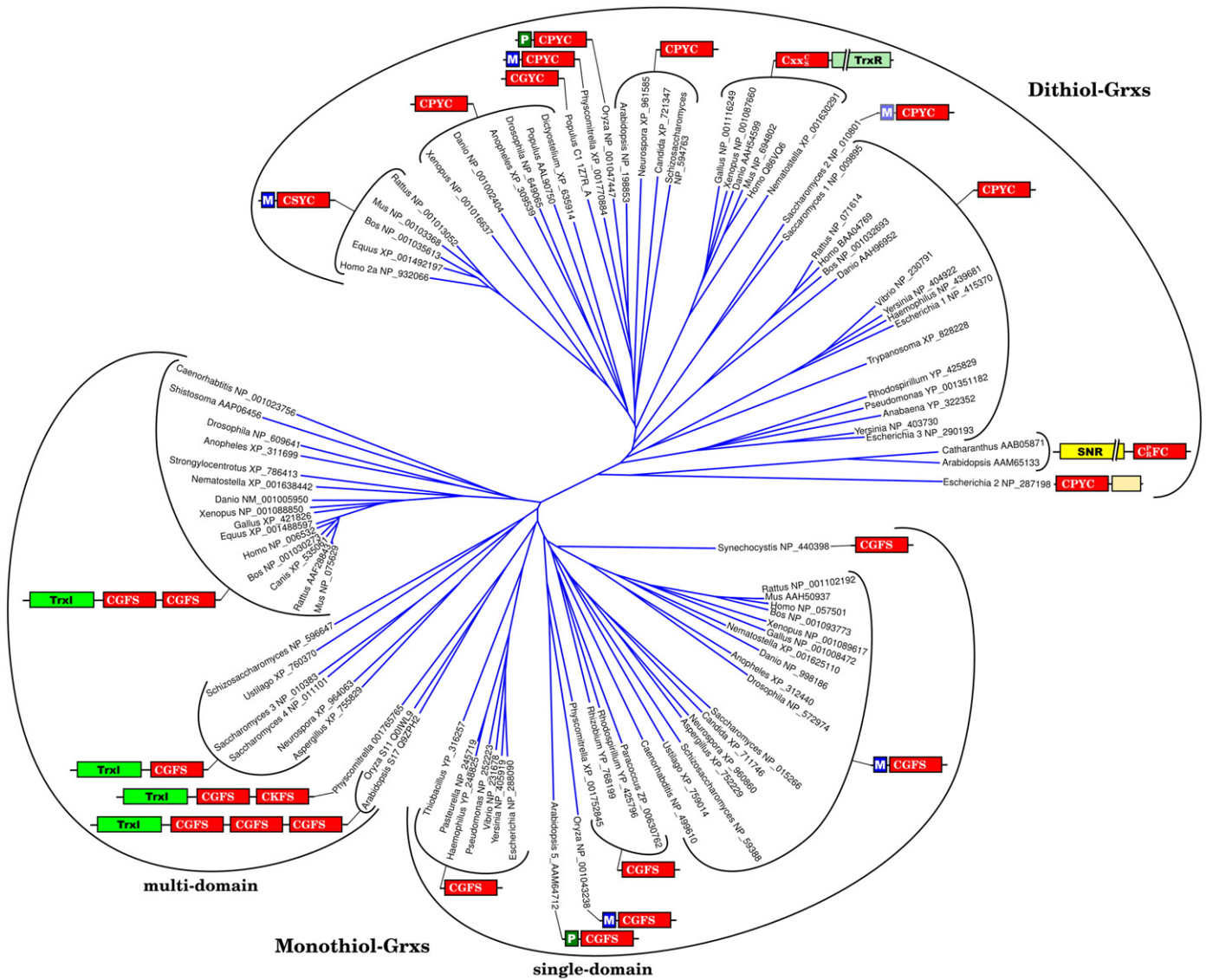
**Fig. 3.** Reaction mechanisms of glutaredoxins. Glutaredoxins catalyze the reversible reduction of protein disulfides utilizing both of their active site cysteinyl residues (reactions 1–4). Disulfides between glutathione and proteins or low molecular weight compounds are reduced in the monothiol mechanism that requires only the more N-terminal active site cysteinyl residue (reactions 6 and 4.). In either case, glutathione disulfide formed in the reaction is reduced by glutathione reductase at the expense of NADPH (reaction 5).

1.4. Enzymatic assays for glutaredoxins

1.4.1. The ribonucleotide reductase assay

This assay is based on the formation of [<sup>3</sup>H]-dCDP from [<sup>3</sup>H]-CDP by class I ribonucleotide reductase (RNR) with electrons from

NADPH via GR, GSH and Grx [1–3]. NADPH consumed during the reaction can be regenerated using glucose 6'-phosphate and glucose 6'-phosphate dehydrogenase. The amount of [<sup>3</sup>H]-dCDP formed in the reaction is determined after hydrolysis to [<sup>3</sup>H]-dCMP and chromatography on Dowex-50 columns by scintillation counting.



**Fig. 4.** Classification of glutaredoxins based on phylogeny, active site and domain structure. The glutaredoxin domain is shown in red including the active sites sequences. Abbreviations used for non-Grx domains: M: mitochondrial signal peptide; P: plastid targeting sequence; SNR: sulfonucleotide reductase; TrxL: thioredoxin like; TrxR: thioredoxin reductase. Details are discussed in the text.

Alternatively, the reaction can be followed spectrophotometrically as consumption of NADPH using unlabeled dCDP as substrate [3]. The reduction of *E. coli* RNR (NrdAB) requires both Grx active site cysteinyl residues [12].

#### 1.4.2. The hydroxyethyl disulfide (HED) assay

The HED assay is arguably the most commonly used Grx-specific enzymatic assay. HED, or  $\beta$ -mercaptoethanol ( $\beta$ -ME) disulfide, as substrate was first introduced to assay GSH-disulfide transhydrogenase activity of Grxs [3,19]. The functional characterization of an *E. coli* Grx1 mutant lacking the more C-terminal active site residue revealed that the preferred substrate in this reaction is not HED itself [12]. During the initial pre-incubation, HED is spontaneously reduced by GSH yielding  $\beta$ -ME and a disulfide between  $\beta$ -ME and GSH ( $\beta$ -ME-SG). Following the addition of Grx to the assay mixture,  $\beta$ -ME-SG is reduced via the monothiol mechanism yielding  $\beta$ -ME and the mixed disulfide between GSH and the more N-terminal active site cysteinyl residue of the Grx. This disulfide is subsequently reduced by a second molecule of GSH yielding GSSG. The reaction can be followed continuously in a spectrophotometer as consumption of NADPH by GR during reduction of the product GSSG.

#### 1.4.3. Reduction of phosphoadenylylsulfate (PAPS) reductase

PAPS reductase activity is measured in an end-point assay as acid-labile sulfite formation from  $^{35}\text{S}$ -PAPS [20].  $^{35}\text{S}$ - $\text{SO}_3^{2-}$  produced in the reaction can be selectively removed from the assay mixture by acidification in form of gaseous  $\text{SO}_2$ . Absorbed by trioctylamine,  $^{35}\text{S}$ - $\text{SO}_3^{2-}$  can be quantified by scintillation counting. In this assay Grxs can be kept in the reduced state by dithiothreitol (DTT), because *E. coli* PAPS reductase does not exhibit background activity with DTT as sole electron donor. Alternatively, PAPS reductase activity can be measured in a coupled optical assay following the reduction of GSSG by GR at the expense of NADPH [21]. Reduction of PAPS reductase requires the dithiol mechanism and is performed with equal efficiency by both Trxs and Grxs [22,23].

#### 1.4.4. Reduction of dehydroascorbate

The Grx-catalyzed reduction of dehydroascorbate to ascorbate by GSH can be followed directly in a spectrophotometer based on the change in absorbance at 265.5 nm [17]. This reaction (for details see previous chapter) is catalyzed by a number of enzymes and therefore not particularly specific for Grxs [18].

Reduction of glutathione mixed disulfides and small molecular weight disulfides – As indicated above, the reduction of any Grx substrate can be followed in a coupled optical assay in which the product GSSG is reduced by GR using electrons from NADPH. Grxs show a very high specificity for GSH-mixed disulfides. Basically any protein or small molecular weight compound can serve as substrate provided that it readily forms a mixed disulfide with GSH, e.g. HED (see above). A number of model substrates have been described, for instance glutathionylated ribonuclease A [24], S-sulfocysteine [19,25] and Cys-SG [19] (see also chapter “Reversible glutathionylation”).

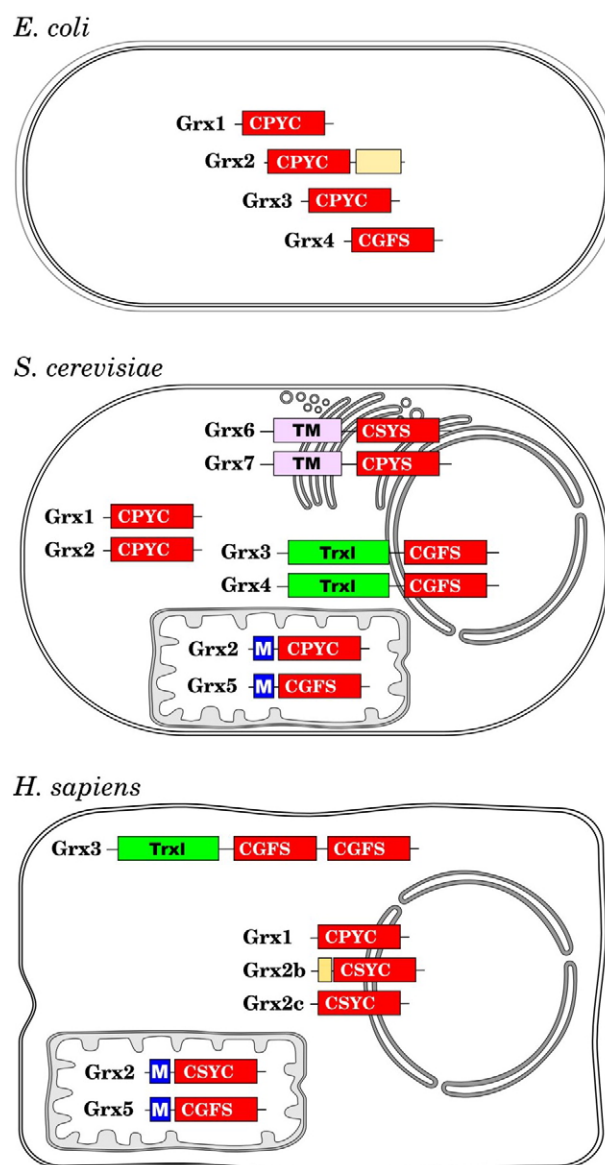
### 1.5. Classification of glutaredoxins

Traditionally, Grxs were named by numbers in order of their discovery in various species. As a result of this, the name of any desired Grx does not indicate to which class it belongs. A new system of classification based on structure, biochemical characterization and cellular function would be helpful.

Based on phylogeny, sequence and domain structure, two main groups of Grxs can be distinguished today (Fig. 4). First, the dithiol Grxs containing the active site consensus sequence Cys-Pro-Tyr-Cys and, secondly, the monothiol Grxs with a Cys-Gly-Phe-Ser consensus active site sequence. Monothiol Grxs can be further categorized into single-domain monothiol Grxs consisting of only one Grx domain and multi-

domain monothiol Grxs that contain a N-terminal Trx-like domain and one to three C-terminal monothiol Grx domains, sometimes also named PICOT homology domains [26]. To avoid further confusion, it is important to note that all dithiol Grxs investigated so far catalyze monothiol mechanism reactions such as the HED assay. Many, but not all of them, catalyze dithiol reactions as well. The most stunning fact about the monothiol Grxs is that most of them lack activity in either type of reaction with all established Grx model substrates. While dithiol Grxs and single-domain monothiol Grxs are ubiquitously present in all kingdoms of life, multi-domain monothiol Grxs are restricted to eukaryotic cells (Figs. 4 and 5). Grx domains are sometimes also part of other proteins. For instance, the group of plant sulfonucleotide reductases contains a C-terminal Grx fusion that serves as internal electron donor accepting electrons from GSH (see below). A subtype of Trx reductases, named thioredoxin glutathione reductases (TGRs) contains a N-terminal Grx domain that is related to the dithiol Grxs (Fig. 4) although the C-terminal active site cysteine is in some cases lost [27].

Grxs represent a rather heterogeneous family of proteins and many organisms contain a unique composition of Grxs (Fig. 5). For instance,



**Fig. 5.** Intracellular distribution and domain structure of glutaredoxins in *E. coli*, *S. cerevisiae*, and *H. sapiens*. The glutaredoxin domain is shown in red including the active sites sequences. Abbreviations used for non-Grx domains: M: mitochondrial signal peptide; TM: transmembrane domain; Trx1: thioredoxin-like domain.

*E. coli* contains four glutaredoxins, two classical dithiol Grxs (Grx1 and Grx3), one unusual dithiol Grx (Grx2) and one monothiol Grx (Grx4) (Figs. 4 and 5, overviews in [28,29] and Vlamis-Gardikas 2008, this special issue). Grx1 can serve as electron donor for metabolic enzymes like RNR and PAPS reductase (see previous chapters), but it is also active in monothiol mechanism reactions. Grx3 cannot normally compensate the loss of Grx1 and its function *in vivo* is still unclear. Grx2 contains an N-terminal Grx domain followed by an alpha-helical domain and is structurally similar to the GSH S-transferases family of proteins. The protein is highly efficient in monothiol-type reactions and resembles the majority of *E. coli*'s GSH-dependent oxidoreductase activity. The monothiol Grx4 does not exhibit classical Grx activity, but it can be reduced by Trx reductase and seems to be involved in iron homeostasis [30,31]. As of today, seven Grxs and at least one more potential Grx-like protein were described in *Sacharomyces cerevisiae* (Fig. 5, [32,33]): the dithiol Grxs 1 and 2, the multi-domain monothiol Grxs 3 and 4, the mitochondrial single-domain monothiol Grx5 and two unusual monothiol Grxs related to the exocytotic pathway that are anchored to the ER/Golgi membrane with their Grx domains facing the luminal site of these compartments [33–35]. Human cells contain four Grxs. The cytosolic dithiol Grx1 is a functional homologue of *E. coli* and yeast Grx1. The mainly mitochondrial Grx2 (Grx2a) contains the active site Cys-Ser-Tyr-Cys. This subtle modification (Ser for Pro) enables the protein to receive electrons from Trx reductase and to complex an iron-sulfur cluster [36,37]. Testicular cells and some cancer cells express two additional cytosolic/nuclear isoforms of the protein (Grx2b and Grx2c) derived from alternative transcription initiation and splicing. One of these isoforms – Grx2b – is not able to coordinate the cluster [38]. Human Grx3 (PICOT/TXNL-2) is a multi-domain monothiol Grx and a homologue of yeast's Grx3 and 4 [26,39]. The mitochondrial single-domain monothiol Grx5 is well conserved amongst eukaryotic cells and thus also present in human cells. In addition, human cells contain a TGR that is predominantly expressed in testes, particularly in elongated spermatids [40]. An intriguingly complex transcription and splicing pattern has been revealed for cytosolic Trx reductase [41–43]. Remarkably, one of these transcript variants also contains a glutaredoxin domain fused to the N-terminus and is primarily expressed in testes [43].

From an evolutionary point of view it is interesting to note that the monothiol Grxs show a higher degree of homology compared to the dithiol Grxs (Fig. 4). As an example of this conservation, mitochondrial monothiol Grxs represent a compact phylogenetic unit that evolved from a common bacterial origin. Mitochondrial dithiol Grxs, in contrast, seem to have evolved multiple times separately from each other, for instance, in mammals and fungi.

## 2. Functions of glutaredoxins

### 2.1. Glutaredoxins as electron donor

Glutaredoxins were first identified for their ability to deliver electrons to RNR [1,2]. RNRs provide the building blocks for DNA synthesis in all organisms by conversion of ribonucleotides to deoxy ribonucleotides (overviews in [44] and [45]). RNRs fall into three major classes: Aerobic prokaryotes and eukaryotes utilize class I enzymes to cover their need for deoxy ribonucleotides. Class I RNRs consist of two subunits, R1 and R2, in an  $\alpha_2\beta_2$  arrangement. Subunit R1 harbors the catalytic center and a redox-active cysteine pair, R2 contains a di-iron center and a stable tyrosyl radical. Class II RNRs, present in aerobic and anaerobic prokaryotes, consist of only one subunit in an  $\alpha$  or  $\alpha_2$  arrangement. These proteins contain adenosylcobalamin as cofactor and use Trx as electron donor. Class III RNRs are homodimeric proteins found in anaerobic prokaryotes. Class III proteins use formate as electron donor and contain a stable glycy radical, whose generation requires a [4Fe-4S]-containing activase (NrdG in *E. coli*).

The catalytic cycle of class I RNRs requires the reduction of a disulfide in their R1 subunit. In *E. coli*, Trx1 and Grx1 can serve as electron donors for the reduction of this disulfide under physiological conditions [3,46] (for a review, see [47]). In a *trxA grxA* null mutant, overexpressed Grx3 can compensate for the lack of reducing equivalents, although the protein exhibits only weak activity with RNR [48]. Using random mutagenesis combined with a genetic screening, Ortenberg et al. identified Grx3 mutants that more efficiently compensated for the lack of Trxs and Grx1 in *E. coli*. Remarkably, all these mutants showed an exchange of Met43, an amino acid located in the core of Grx3 [49]. The most effective mutant (Met43Val) was able to reduce NrdAB much more efficiently than the wild-type protein *in vitro* with a  $V_{max}$  close to that of Grx1. The Met43Val substitution lowered the redox potential of Grx3 by 11 mV, presumably by lowering the pKa of the N-terminal active site thiol [21]. The kinetic constants of *E. coli* NrdAB with Trx1 and Grx1, the levels of the redoxins and thymidine incorporation experiments in different mutant strains suggest Grx1 to be the main electron donor for NrdAB [50,51]. Hence, it came as a surprise when a second class I (Ib) RNR (NrdEF) was identified [52]. The function of this enzyme in bacteria containing both NrdAB and NrdEF remains mysterious, because NrdEF cannot compensate for the lack of NrdAB. In contrast to NrdAB operons, NrdEF operons often encode for a specific electron donor (NrdH). This protein of approx. 10 kDa is a Grx-like protein with the activity profile of a Trx [53]. Structurally, NrdH is most similar to *E. coli* Grx3 and phage T4 Grx, however, it lacks the GSH binding site and is reduced by TrxR [54]. *In vitro*, Grx1, but not Trx1, can replace NrdH as electron donor for NrdEF [53].

The yeast genome encodes two class I RNR R1 subunits, Rrn1 and Rrn3 [45,55]. The functional RNR contains primarily Rrn1 and can use both Trxs and Grxs as electron donors. Deletion of the two genes encoding cytosolic Trxs gave rise to a viable strain, however, with reduced growth rate due to an elongated S and a shortened G1 phase [56]. In these mutant cells, the dNTP pools were reduced to about 60% compared to wildtype [57], indicating a physiological role of both Trxs and Grxs as electron donor for ribonucleotide reduction in yeast.

Mammalian cells contain two genes encoding R2 RNR subunits that share a common R1 subunit as interaction partner. R2 is the enzyme that provides the building blocks for DNA synthesis during cell division, p53R2, a downstream target of the tumor suppressor p53, is believed to play a role in DNA repair [45]. Mammalian Trxs (Trx1) and dithiol Grxs (Grx1) are efficient substrates for their endogenous R1 subunits *in vitro* [58][Zahedi Avval and Holmgren, unpublished results]. Their importance as electron donor *in vivo*, however, is less clear. Among human tissues, testes are one of the places with the highest proliferation rate. Remarkably, spermatogonia, which give rise to the whole line of sperm cell development, show intense staining for RNR subunit R1 but not for Trx1 or Grx1 [59–61].

Bacteria, fungi and plants utilize inorganic sulfate as source for the synthesis of reduced sulfur compounds such as cysteine, methionine, and many cofactors. Reduction of sulfate ( $SO_4^{2-}$ ) to sulfide ( $S^{2-}$ ) requires eight electrons and takes place in two steps. First, sulfate is activated to adenylylsulfate (APS) or PAPS and subsequently reduced to sulfite ( $SO_3^{2-}$ ) by APS and PAPS reductase, respectively. Secondly, sulfite is reduced to sulfide using six electrons provided by NADPH in bacteria and fungi or ferredoxin in photosynthetic organisms. The requirement for a low molecular weight dithiol reductant in the first step was originally described by Wilson et al. [62]. In parallel to the history of ribonucleotide reductase, Grx was identified to be the alternative electron donor for sulfate reduction in an *E. coli* mutant lacking Trx [63].

Bacterial-type APS and PAPS reductases act in a ping-pong mechanism. The proteins contain a single catalytic cysteinyl residue in their active site that, upon reduction of the substrate, becomes oxidized to form a disulfide between two monomers [64]. This disulfide is reduced in a dithiol reaction mechanism by Trxs and/or

Grxs, for instance, in *E. coli* [22], *Bacillus subtilis* [65], and yeast [66,67]. In addition to their role as electron donor, *E. coli* Grxs were shown to regulate PAPS reductase activity through reversible S-glutathionylation of the active site cysteine in response to oxidative challenges [23] (see chapter “Reversible glutathionylation”). Plant-type APS reductases contain a C-terminal extension in form of a single dithiol glutaredoxin domain containing the active site sequence CPFC or CRFC [68–70]. This direct association of the sulfonucleotide reductase with a Grx domain enables the plant-type enzymes to directly use electrons provided by GSH. The Grx domains of plant-type sulfonucleotide reductases have been reported to be active in both dithiol reactions, such as the reduction of insulin, as well as monothiol reactions, for instance catalyzing the HED assay and the reduction of dehydroascorbate [71,70].

Resistance to arsenate in *E. coli* is conferred by the *ars* operon carried on plasmid R773. This operon includes the *arsC* gene. The ArsC protein catalyzes the reduction of arsenate to arsenite. This activity requires a Grx as a source of reducing equivalents and *E. coli* Grx2 was shown to be the most effective hydrogen donor for the reduction of arsenate by ArsC [72,73].

In plants with their great variety of Grxs, additional functions of Grxs as electron donor for peroxide, methionine sulfoxide, and even Trx reduction have been demonstrated. For details we refer to the excellent overviews provided in the following references: [74] and Rouhier et al. 2008 (this special issue).

## 2.2. Reversible glutathionylation

Oxidative stress broadly impacts cells, initiating a series of redox-dependent modifications of proteins, lipids and nucleic acids. With respect to proteins, cysteinyl residues are of particular interest, because their thiol group (P-SH) is susceptible to a number of oxidative modifications. Inter- or intra-molecular disulfides can be formed between neighboring protein thiols (P-S-S-P) or between protein thiols and low molecular weight thiols such as GSH (S-glutathionylation, P-S-SG). The reaction with reactive oxygen and nitrogen species may lead to sulfenic (P-SOH), sulfinic (P-SO<sub>2</sub>H) and sulfonic (P-SO<sub>3</sub>H) acid as well as S-nitroso groups (S-nitrosylation, P-S-NO). Countless examples have been reported where these types of modifications alter the function of proteins containing cysteines of structural importance, as part of protein-protein interaction interfaces or within their active site. There is upcoming evidence, that the reversible formation of mixed disulfides of protein thiols with GSH is a key mechanism in redox regulation and signaling comparable to reversible protein phosphorylation. Glutaredoxins catalyze both the formation and the reduction of mixed disulfides between protein thiols and GSH [75,76]. In general, the reduction of these mixed disulfides is favored, but under conditions where the concentration of GSH is decreased and GSSG is increased, mixed disulfides may also be formed [76]. For a detailed overview of this topic and the involvement of Grxs in different species we refer to some of the following overview articles: [74,77–81].

In brief, in human cells glutathionylation has been shown to regulate a number of key proteins and processes in response to alterations in the redox state, for instance actin polymerisation [22], glyceraldehyde 3-phosphate dehydrogenase [82], protein tyrosine phosphatase 1B [83], creatine kinase [84], c-Jun [85], NF $\kappa$ B subunit p50 [86], caspase-3 [87], and HIV protease [88]. Many of these studies indicate that human Grx1 can catalyze the reduction of the mixed disulfides, regenerating the activities of these proteins [82,83,88,87]. Human Grx2 may regulate the activity of mitochondrial membrane proteins, such as complex I, by reversible glutathionylation [89].

In plants, GSH is involved in fundamental processes in plants like flowering [90], root hair density and length [91], trichoblast cell length [91], or the G<sub>1</sub> to S phase transition [92]. Plant proteins found to

undergo reversible glutathionylation include glutathione S-transferases [93], mitochondrial isoform of thioredoxin h [94], chloroplastic f-type Trxs [95], chloroplastic glyceraldehyde 3-phosphate dehydrogenase [96], and soybean protein tyrosine phosphatase [97]. Many more targets for glutathionylation were suggested by an *in vivo* and *in vitro* proteomic approach using biotinylated GSSG to label sensitive targets [98]. 79 proteins, including GAPDH and fructose-1,6-bisphosphate aldolase, that can be glutathionylated or associated with glutathionylated proteins were identified. Glutathionylation of dehydroascorbate reductase, zeta-class glutathione transferase, nitrilase, alcohol dehydrogenase, and methionine synthase were confirmed using recombinant expressed and purified proteins [97].

So far, only few glutathionylated proteins were identified in yeast: enolase and alcohol dehydrogenase, and two of the three isoforms of GAPDH [99,100]. One of the isoforms is irreversibly inhibited, whereas activity of the other isoform is restored after oxidative stress [100]. This isoform can be de-glutathionylated by the monothiol Grx5 [101]. In a yeast mutant lacking Grx5 GAPDH glutathionylation was increased and recovery of enzyme activity was inhibited [101].

*Trypanosoma brucei*, responsible for african sleeping sickness, contains alternative low molecular weight thiols, i.e. trypanothione and glutathionyl-spermidine (see Comini et al. 2008, this special issue). However, small amounts of host-derived GSH are present in these parasites. Treatment of recombinant *T. brucei* proteins monothiol Grx1, tryparedoxin peroxidase III, and thioredoxin with GSSG led to specific, reversible glutathionylation [102]. Monothiol *T. brucei* Grx1 does not form a mixed disulfide of GSH with its active site thiol, instead, the non conserved Cys<sup>181</sup> can be glutathionylated leading to the formation of a disulfide bridge between this cysteine residue and the active site cysteine [102].

*E. coli* transcription factor OxyR can undergo several stable, posttranslational modifications of the single regulatory thiol (SH), including nitrosylation, oxidation and glutathionylation *in vivo*. These modified forms of OxyR are transcriptionally active but differ in structure, cooperative properties, DNA binding affinity, and promoter activities. These variations allow fine-tuned differentiated responses to redox signals [103].

## 2.3. Iron metabolism

Iron is an essential element to life present in a number of cofactors including hemes and iron-sulfur centers. Free ferrous iron, however, is an efficient catalyst of Fenton-type reactions generating hydroxy radicals from peroxides. This highly reactive oxygen species reacts with various organic groups damaging proteins, lipids and nucleic acids. To limit this damage, organisms evolved a number of regulatory circuits tightly controlling the levels of intracellular iron. Iron dysregulation is causatively involved in the pathophysiology of various human diseases, for instance Alzheimer's disease [104,105], Friedreich's Ataxia [106,107], hemochromatosis [108,109] and Parkinson's disease [110,111].

### 2.3.1. Iron-sulfur cluster assembly

In eukaryotic cells, mitochondria are essential for the maturation of cellular iron-sulfur proteins [112]. Iron-sulfur cluster synthesis is thought to take place on the scaffold protein Isu (IscU or NifU in bacteria) from where the [Fe-S] units are transferred to apo [Fe-S] proteins with the help of DnaK and DnaJ type chaperons (overviews in [112] and [113]). Yeast mutants lacking mitochondrial monothiol Grx5 are highly sensitive to oxidative damage and osmotic stress. The mutants display an increase in total protein carbonyl content and the oxidation of a number of specific proteins, including transketolase [114]. Moreover, knock-out of yeast Grx5 led to iron accumulation in the cell and inactivation of iron-sulfur containing enzymes [115]. These

defects could be suppressed by overexpression of proteins involved in [Fe–S] assembly, namely the Hsp70/DnaK type chaperon Ssq1 and the potential alternative scaffold Isa2. Hence, a function of Grx5 in iron–sulfur cluster synthesis or repair was suggested [115]. Muhlenhoff et al. demonstrated that depletion of Grx5 from yeast cells led to an increase in the amount of iron loaded scaffold Isu1. This result implies that Grx5 is required in a step following [Fe–S] cluster synthesis on Isu1 when the pre-build clusters are inserted into apo-proteins [116]. Structural bioinformatics predicted the formation of strong and specific complexes between Grx5 and several components of the yeast ISC machinery [117,118]; two-hybrid analysis indicates interaction between Grx5 and Isa1 [117]. A hypochromic anaemia mutant of zebrafish (Shiraz) was recently shown to be caused by deficiency in the zebrafish homologue to yeast Grx5, causing impaired [Fe–S] cluster assembly and as result defects in hem biosynthesis [119]. A human counterpart of the zebrafish Shiraz mutant, caused by a homozygous silent mutation in the human Grx gene that interferes with intron I splicing, showed sideroblastic-like microcytic anemia and iron overload [120]. Grx5 homologues from various species including *E. coli*, *Synechocystis*, *Arabidopsis thaliana*, zebrafish and human rescue the phenotype of the yeast Grx5 mutant when targeted to mitochondria [119,121,122], indicating that the role of monothiol Grxs in [Fe–S] assembly is conserved throughout evolution. Recently, Bandyopadhyay et al. have shown *in vitro* that plastidic plant monothiol Grxs can act as scaffold in the transfer of iron–sulfur clusters from the synthesis machinery to target apo-proteins [123]. However, *in vivo* evidence for this reaction in plastids has not been provided and in yeast Grx5 but not GSH is required for the maturation of mitochondrial [Fe–S] proteins [115,124]. The exact biochemical function of single-domain monothiol Grxs in the synthesis of [Fe–S] proteins remains to be established.

### 2.3.2. Iron homeostasis and glutaredoxins

The ferric uptake regulator (Fur) is the main sensor of iron in *E. coli* and many other bacteria. Loaded with ferrous iron, Fur acts as repressor of genes related to iron uptake and homeostasis [125]. In a *fur*<sup>−</sup> strain of *E. coli* the levels of monothiol Grx4 were slightly increased. Depletion of iron from the cells, especially in the case of the *fur*<sup>−</sup> strain, caused dramatic elevation in levels of Grx4. These data suggest a potential involvement of Fur in transcriptional control of Grx4 and that Grx4 may be involved in pathways depending on iron [30].

In *Saccharomyces cerevisiae*, the expression of genes involved in iron homeostasis is regulated by Aft (activator of ferrous transport) transcription factors (overviews in [126] and [127]). Under iron-sufficient conditions these proteins are localized in the cytosol, upon iron deprivation the proteins shuttle to the nucleus to activate transcription of iron regulon genes. Shuttling between cytosol and nucleus is important for normal function of Aft1. The mechanism of iron sensing by Aft1 is unknown, however, this function depends on a functional mitochondrial iron–sulfur cluster assembly machinery [128]. Two recent studies pointed out a critical role of yeast Grxs 3 and 4 for iron inhibition of Aft1 in yeast cells. These two multi-domain Grxs consist of a N-terminal Trx domain and one C-terminal monothiol Grx domain. Ojeda et al. have shown that cells lacking both Grx3 and Grx4 show constitutive expression of iron regulon genes, while overexpression of Grx4 attenuates wild type Aft1 activity. The thioredoxin-like domain in Grx3 and Grx4 was dispensable in mediating iron inhibition of Aft1 activity, whereas the monothiol glutaredoxin and its Cys–Gly–Phe–Ser active site cysteinyl residue were essential for this function. The direct interaction between Grx3 and Grx4 with Aft1 was demonstrated by both two-hybrid analysis and co-immunoprecipitation [129]. Pujol-Carrion et al. provided additional evidence for a functional protein complex between Grx3, Grx4 and Aft1. The absence of both Grx3 and Grx4 caused an enrichment of G1 cells and a slow growth phenotype. As a consequence of dysregulated iron homeostasis,

*grx3-grx4*<sup>−</sup> cells were highly sensitive to oxidative stress induced by hydrogen peroxide and t-butyl hydroperoxide but, noteworthy, not to oxidation by diamide [130].

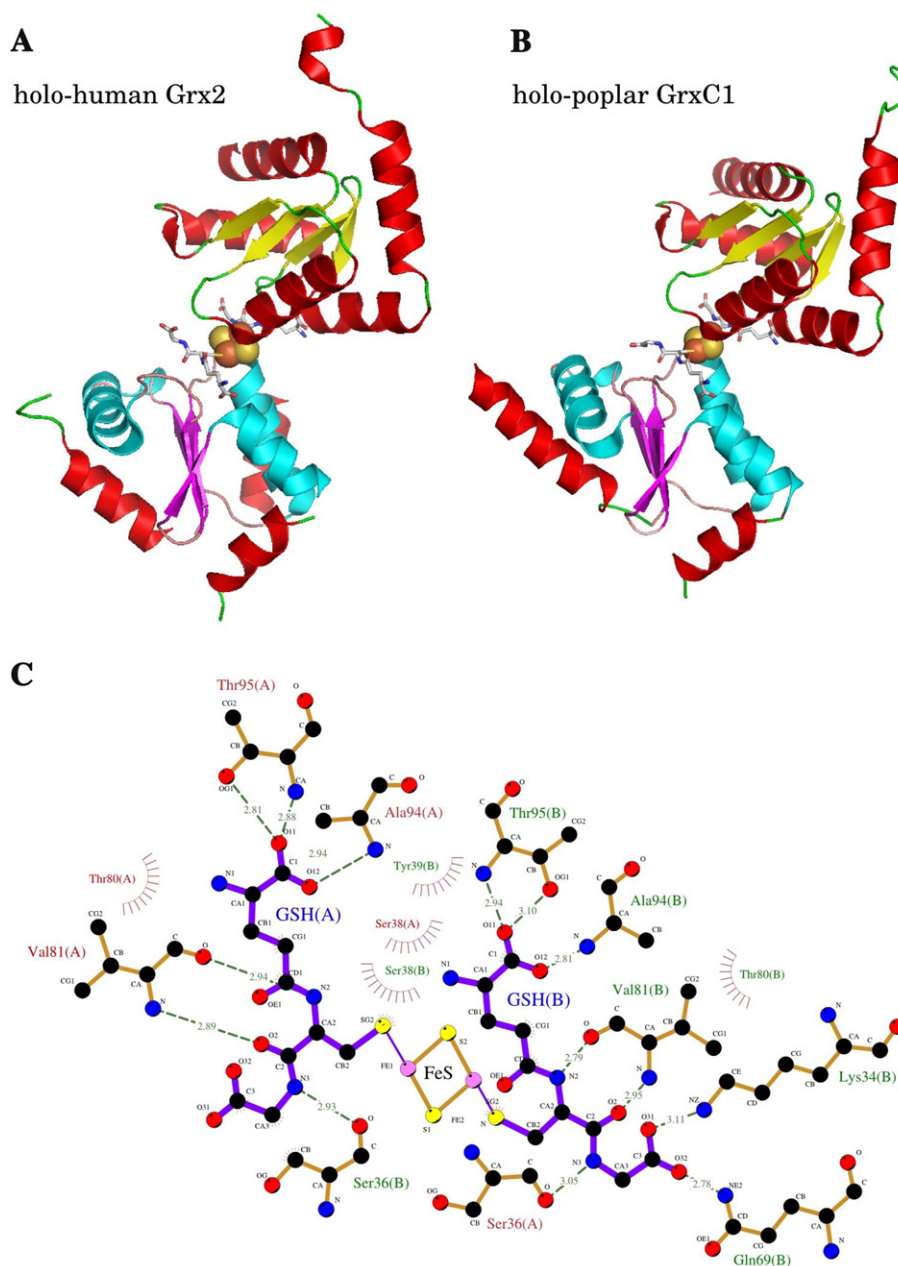
In contrast to yeast, vertebrate cells evolved a posttranscriptional mechanism for the regulation of expression of proteins involved in iron homeostasis and iron cofactor biosynthesis (reviewed, for instance, in [131–133]). Upon iron deprivation, iron regulatory proteins (IRP) 1 and 2, both homologous to mitochondrial aconitase, bind to iron regulatory elements (IRE), hairpin structures present in the mRNA of IRP regulated genes. Translation of mRNAs containing an IRE in their 5'UTR is repressed, while mRNAs containing IRE structures in their 3'UTR are stabilized upon IRP1 and IRP2 activation. Loss of Grx5 in the zebrafish Shiraz mutant impaired mitochondrial [Fe–S] cluster assembly and promoted activation of IRP1. To some extent, knock-down of IRP1 restored hemoglobin synthesis in the Grx5 mutant, demonstrating a crosstalk between hemoglobin production and the mitochondrial [Fe–S] cluster assembly machinery [119].

### 2.3.3. Iron–sulfur cluster containing glutaredoxins

Human mitochondrial Grx2 was identified as the first [Fe–S] Grx [37]. This, in many aspects unusual Grx (active site Cys–Ser–Tyr–Cys), contains a redox inactive [2Fe–2S]<sup>2+</sup> cluster that bridges two Grx2 molecules to form the dimeric holo Grx2 complex. The [Fe–S]-bridged dimer lacks enzymatic activity, but degradation of the cluster and dissociation of the holo complex activates the protein. Slow degradation of the complex under aerobic conditions is efficiently prevented by GSH. GSSG and other redox-active compounds promote cluster degradation and thereby activation of Grx2 [37]. The biochemical analysis of several mutants demonstrated that the iron–sulfur cluster is complexed by the two N-terminal active site thiols of two Grx2 monomers and two molecules of glutathione that are bound non-covalently to the proteins and in equilibrium with glutathione in solution [134]. The structure of the dimeric holo Grx2 complex was solved by X-ray diffraction [135] (Fig. 6A). Astonishingly, hardly any direct molecular interactions between the two protein monomers can be identified (Fig. 6C). Besides of one hydrogen bond and two small hydrophobic interactions, all molecular interactions contributing to the holo complex involve the GSH molecules. The two GSH molecules efficiently shield the iron from the solvent. Only one of the sulfur atoms of the [Fe–S] cluster is solvent exposed. Hence, the [2Fe–2S] cluster may not be able to react with redox compounds that require direct molecular interactions with iron such as hydrogen peroxide. Similar to human Grx2, cytosolic GrxC1 from poplar (Cys–Gly–Tyr–Cys active site) exists as a dimeric iron–sulfur containing holo protein or as a monomeric apo protein in solution. Biochemical and structural analysis demonstrated essentially the same holo complex consisting of a subunit-bridging [2Fe–2S] cluster that is ligated by the catalytic cysteines of two Grxs and the thiols of two GSH molecules [136,137] (Fig. 6B).

How and why do some Grxs incorporate an iron–sulfur cluster? The properties that permit Grx2, GrxC1 and other Grxs to form the [Fe–S] bridged dimeric holo complex are likely due to the exchange of the active site Pro. This exchange allows a higher flexibility of the main chain in the active site area providing enough room for the non-covalent binding of GSH and cluster coordination [135,137]. In fact, when the active site of human Grx1 (active site Cys–Pro–Tyr–Cys), which normally cannot bind the cluster, was changed to the corresponding Cys–Ser–Tyr–Cys sequence of Grx2, Grx1 became able to complex the [2Fe–2S] cluster as well [134]. Mutagenesis on a variety of poplar glutaredoxins suggests that the incorporation of an iron–sulfur cluster could be a general feature of plant glutaredoxins possessing a glycine residue adjacent to the catalytic cysteine instead of a proline [137].

Iron–sulfur centers are multipurpose structures found in all forms of life. They can undergo redox reactions, influence protein folding, and act as catalytic centers [138,139]. The vulnerability of [Fe–S]



**Fig. 6.** Iron-sulfur cluster binding in glutaredoxins lacking the active site prolyl residue. (A) Structure of holo human Grx2 (PCB accession number: 2HT9). (B) Holo-poplar GrxC1 (2E7P). (C) Molecular interactions between the two protein subunits, glutathione and the 2Fe<sub>2</sub>S cluster in the structure of human Grx2.

centers to oxidative destruction is sometimes used in sensing and regulatory functions [140]. Co-immunoprecipitation of <sup>55</sup>Fe with human Grx2 from two different cell lines strongly advocated for the presence of the [Fe–S] cluster *in vivo* [37]. We therefore proposed that Grx2's iron–sulfur cluster serves as redox sensor for the activation of the protein during conditions of oxidative stress. When free radicals are formed and the glutathione pool becomes oxidized, reduced glutathione may become the limiting factor for cluster coordination, leading to dissociation of the holo complex and enzymatically active Grx2 [37,134]. Recent data indicate that, at least *in vitro*, many monothiol Grxs (active site Cys–Gly–Phe–Ser) may form the same holo [Fe–S] complex described for human Grx2 and poplar GrxC1, pointing to a possible role of [Fe–S] Grxs in cellular iron metabolism [34,141]. Although the discovery of iron–sulfur Grxs undoubtedly represents a milestone for the Grx research field, at present, the molecular functions of these clusters remain elusive.

### 3. Glutaredoxins in health and disease

#### 3.1. Infection and the immune system

Grxs play an essential role in the life cycles of many viruses as well as in host-virus interactions [142]. Phage T4 and orthopoxviruses like vaccinia, ectromelia, and smallpox encode their own Grxs [143,144], which are essential for DNA synthesis, disulfide bond formation and virus assembly [145–147]. Human Grx1 was detected both within and on the surface of HIV particles. Grx1 can regulate the activity of HIV-1 protease *in vitro*, and could therefore be important for the regulation of protease activity in infected cells [88].

Grx3/PICOT was first identified in a two-hybrid screen aiming at the identification of protein kinase C (PKC)-interacting proteins [39]. In this initial study transient overexpression of Grx3/PICOT inhibited

the activation of c-Jun N-terminal kinase and transcription factors AP-1 and Nf- $\kappa$ B in Jurkat T cells stimulated with anti-CD3/CD28 antibodies, phorbol myristate acetate, or UV radiation. This effect was dependent on both the Trx and Grx domains of Grx3/PICOT, while the Trx domain alone was sufficient for the interaction of Grx3/PICOT with PKC. Full-length Grx3/PICOT displayed some selectivity with respect to its ability to associate with different (overexpressed) PKC isoform in Jurkat T cells. GST pull-down assays using Grx3/PICOT as bait demonstrated binding to PKC, to a lower extent also to PKC $\zeta$ , but not to PKC $\alpha$  [39]. In a subsequent study, treatment of Jurkat T cells with hydrogen peroxide was reported to induce tyrosine phosphorylation of Grx3/PICOT in a dose-dependent manner directly or indirectly dependent on lymphocyte protein tyrosine kinase. Thus, a role of Grx3/PICOT in cell activation-associated signaling pathways or in the cellular response to stress signals was proposed [148].

As outlined above many organisms contain a unique composition of redox enzymes including Grxs. In the case of pathogens, these differences might be utilized for therapeutic purposes in the future. For in depth discussions, see for instance [149–151], and Comini et al. and den Hengst et al. 2008 (this special issue).

### 3.2. The airway system

The glutathione redox couple plays a crucial role in protecting the lung against exogenously as well as endogenously induced oxidative stress [152,153]. Cigarette smoke increases the amount of reduced GSH in the epithelial lining fluid of smokers [154] and in the intracellular space of exposed animals [155], whereas it is decreased in asthma patients [156].

The expression of the two dithiol Grxs, Grx1 and Grx2, in lung tissues was demonstrated both on mRNA and protein level [38,61,157–160]. In mouse the basal levels of Grx2 in lung tissue are higher than those of Grx1 [158,160], in human Grx1 exceeds the levels of Grx2 [159]. Also the localization differs between human and other mammalian species. In mouse and calf Grx1 is mainly present in the airway epithelium [61,160], whereas in human Grx1 is highly expressed in alveolar macrophages [159]. The number of these alveolar macrophages is significantly increased in the lung tissues of smokers [161]. During the progression of chronic obstructive pulmonary disease (COPD) the number of Grx1 containing macrophages decreases in correlation with functional lung parameters [161]. In acute COPD significant higher Grx1 levels were detected in sputum. Therefore Peltoniemi et al. proposed a role of Grx1 in reduction of extracellular GSH-mixed disulfides during oxidative stress in COPD [161]. In addition, decreased levels of Grx1 were found in alveolar macrophages of patients with sarcoidosis and allergic alveolitis, but not with interstitial pneumonia [159]. The effect of endogenous stimuli on the expression level of Grx1 in lung tissue is not clear. In a first microarray study comparing smokers and non smokers no difference in Grx1 mRNA levels were detected [162], while a second study reported a 10-fold increase in Grx1 levels 5–10 hours after exposure to cigarette smoke [163]. In allergic airway diseases Grx1 but not Grx2 expression is increased along with a low level of S-glutathionylated proteins [160].

### 3.3. The cardiovascular system

Various aspects of the physiological and disease-related functions of the Grx systems in the cardiovascular systems have been discussed before [164–167]. The aim of this section is to provide an update of the most recent developments not reviewed before.

Recently, two studies reported the effect of the knock-out of Grx1 on myocardial ischemia-reperfusion injury in mice [168,169]. The first study reported that loss of Grx1 did not sensitize adult mice

to ischemia/reperfusion-induced injury or hypoxia, although embryonic fibroblasts were sensitized to oxidative stress [168]. The second study described that Grx1 deficiency depressed functional recovery and increased infarct size in coronary occlusion/reperfusion models of heart infarction and increased ROS production during ischemia and reperfusion. Overexpression of Grx1 in the heart resulted in reduced ROS production during ischemia and reperfusion [169].

Myocardial overexpression of mitochondrial Grx2 (Grx2a) in the heart could rescue the cardiac cells from apoptosis and necrosis induced by ischemia and reperfusion. Isolated hearts from these transgenic mice displayed significantly improved contractile performance and reduced myocardial infarct size and cardiomyocyte apoptosis. Loss of cardiolipin, cytochrome c release and activation of both caspase 3 and caspase 9 was attenuated and the GSH/GSSG ratio was preserved. Grx2a mediated survival of cardiomyocytes involved PI-3-kinase-Akt survival signaling pathway and the activation of NFB and Bcl-2 [170].

Serum response factor is a transcription regulator essential for the formation of mesoderm tissue and thereby for cardiac development. Zhang et al. have used a chromatin immunoprecipitation assay to identify targets regulated by serum response factor in a dimethyl sulfoxide-induced P19 cardiac cell differentiation model. In this study, Grx3/PICOT (TXNL2) was verified as direct target of serum response factor, implying a role of this monothiol Grx in the early embryonic development of cardiac tissue [171]. Cardiac hypertrophy is an adaptive response of myocardial tissue to a variety of pathological conditions and significantly increases the risk for sudden death caused by heart failure. Various signaling pathways have been implied in the development of hypertrophy, many of which include PKC isoforms [172]. Jeong et al. have analyzed a possible role of Grx3/PICOT in the development of cardiac hypertrophy [173]. Grx3/PICOT expression was found to be upregulated in hypertrophic adult rat hearts induced by transverse aortic constriction as well as in neonatal rat cardiomyocytes after exposure to endothelin-1 (ET-1) or phenylephrine (PE). Overexpression of Grx3/PICOT in neonatal cardiomyocytes inhibited the hypertrophic response after treatment with ET-1 or PE. Mice transgenically overexpressing Grx3/PICOT in cardiomyocytes exposed to transverse aortic constriction displayed a significantly blunted increase in the heart to body weight ratio compared to wild-type litter mates. Noteworthy, Grx3/PICOT overexpression reduced the induction of several fetal genes associated with cardiac hypertrophy. Moreover, cardiomyocytes isolated from the transgenic mice showed an approx. 90 % increase in contractility and significantly improved ventricular function likely to be coupled to a more efficient re-uptake of Ca<sup>2+</sup> to the sarcoplasmic reticulum [173]. Recently, Jeong et al. demonstrated the direct interaction of Grx3/PICOT's Grx domain with muscle LIM protein (MLP) [174]. MLP is critical for calcineurin-NFAT (nuclear factor of activated T cells) signaling. In fact, Grx3/PICOT negatively affects calcineurin-NFAT signaling through its interaction with MLP. Interestingly, the N-terminal Trx-like domain of Grx3/PICOT is dispensable for the inhibitory effect of Grx3/PICOT on cardiac hypertrophy.

The role of the mitochondrial monothiol Grx (Grx5) in iron homeostasis and the severe effects of loss-of-function mutants with respect to heme synthesis and anemia have been discussed in previous chapters.

### 3.4. The nervous system

The distribution of the four different Grx systems in the vertebrate/mammalian brain have not been analyzed in detail so far. However, convincing evidence for a role of Grxs in oxidatively stressed neurons does exist. Oxidative damage is strongly associated with the loss of neurons in several neurodegenerative diseases like Alzheimer's disease (AD), amyotrophic lateral sclerosis or Parkinson's disease (PD)

as well as with neuronal cell death following an ischemic insult. It cannot be distinguished whether oxidative stress is the primary cause or the consequence of neuronal cell death, however, the beneficial effects of oxidative stress response proteins have been demonstrated repeatedly, making them prime candidates for the development of new therapeutic strategies [175].

Induction of cerebral ischemia in rodents is a common model for hypoxia-reperfusion injury. Following middle cerebral artery occlusion, Grx1 expression was shown to be reduced in areas with neuronal damage [176]. It remains to be proven that timely restoration of Grx1 levels in these areas could be beneficial during focal ischemia.

Expression profiling of expressed sequence tagged complementary cDNAs in single tangle-bearing versus normal CA1 neurons aspirated from sections of AD and control brains, revealed the reduction of Grx1 mRNA levels in the tangle-bearing neurons [177]. Corroboratively, increased Grx1 protein levels were demonstrated in AD brain samples [178]. Oxidation of Grx1 by amyloid- $\beta$  treatment and the attenuation of amyloid- $\beta$  toxicity by overexpression of both Grx1 was demonstrated in dopaminergic SH-SY5Y neuroblastoma cells. Thus, amyloid- $\beta$  toxicity might be mediated, at least in part, by oxidation of Grx1 and subsequent induction of apoptosis, for instance via activation of ASK1 [178]. PD is characterized by loss of dopaminergic neurons in the substantia nigra causing break-down of the nigro-striatal pathway. An overwhelming body of evidence documents the importance of both redox and iron homeostasis in PD, e.g. in form of the loss and oxidation of the GSH pool [110]. Dopaminergic neurons are especially vulnerable due to the propensity of dopamine to auto-oxidize and thereby produce elevated levels of hydrogen peroxide. Human Grx1 and *E. coli* Grx2, administered to the medium, have been shown to protect cerebellular granule neurons from dopamine-induced apoptosis by activating NF- $\kappa$ B via Ref1 [179] involving the Ras-phosphoinositide 3-kinase and JNK pathways [180], supporting a protective role of Grxs in PD. The emerging functions of Grxs in the control of redox and iron homeostasis imply various potential roles in the pathology and therapy of PD. It remains to be investigated if and how the different Grx systems are involved in the etiology of PD and other neurodegenerative diseases.

The role of Grxs and the GSH system in protecting the lens of the eye against exogenous and endogenous oxidative stress have been reviewed in detail before [181,182].

### 3.5. Reproduction

The constant cell division and DNA synthesis in the seminiferous tubuli of the testis require a constant supply of deoxyribonucleotides by RNR. Surprisingly, neither Trx1, nor Grx1 co-localize with RNR in rat and calf testes, where RNR is primarily localized in the highly proliferative spermatogonia cells [59,61]. Prominent staining of Grx1 was detected in Sertoli cells and weak staining in Leydig cells. These two cell types support spermatogenesis at different levels, but do not proliferate themselves. Recently, we demonstrated the presence of two non-mitochondrial isoforms of human Grx2 in testis [38]. Grx2-specific immunolabeling was detected in spermatogonia, Sertoli cells and in both round and elongate spermatids. One might therefore speculate about a function of cytosolic Grx2 as electron donor for RNR in spermatogonia, future research will address this question.

Grx1 is induced during pregnancy [183] and may be involved in the regulation of cervical ripening, particularly following prostaglandin E2 treatment, which is the most commonly used substance for cervical priming and induction of labour.

Pre-eclampsia is one of the major contributors to perinatal morbidity. Grx1 expression is affected in placenta from pregnancies with pre-eclampsia and/or growth restriction of fetuses, and the decrease in expression correlates to the severity of the condition [184]. Oxidatively modified proteins accumulated to a greater extent in pre-eclamptic placentae compared to normal placentae. In both normal

and pre-eclamptic placentae, Grx1 was detected in the trophoblasts of the floating villi. The levels of the protein were increased approximately 2 to 3-fold in the pre-eclamptic placentae compared to controls, suggesting that the protein might function in protecting placental functions against oxidative stress caused by pre-eclampsia [185].

### 3.6. Apoptosis and cancer

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein (MAP) kinase kinase kinase that activates the c-Jun N-terminal kinase (JNK) and the p38 MAP kinase pathways and is required for tumor necrosis factor  $\alpha$ -induced apoptosis [186]. Similar to Trx1 [187], human Grx1 binds to ASK1 dependent on its redox status. In this complex the kinase activity of ASK1 is suppressed. Oxidation of Grx leads to dissociation of the complex and activation of ASK1 [188]. Hence, Grx1 may regulate ASK1's kinase activity in response to the glutathione redox state [189]. Grx1 protects cells from hydrogen peroxide-induced apoptosis by regulating the redox state of protein kinase B (Akt) [190,191]. Grx1 was also implied in caspase-3 activation via reversible glutathionylation of the protein. Grx1 activity is significantly upregulated by tumor necrosis factor- $\alpha$  in endothelial cells and siRNA knock-down of Grx1 significantly inhibited tumor necrosis factor- $\alpha$ -induced endothelial cell death due to attenuated caspase-3 cleavage concomitant with increased caspase-3 glutathionylation [192].

Overexpression of Grx1 increases the resistance of MCF7 breast cancer cells to doxorubicin, a widely used anti-cancer agent [193]. Multiple applications of protein kinase C activating phorbol esters increases the activity of Grx1 and the Trx system in mouse skin for several days. These activations may play a general role in the epigenetic mechanism of tumor promotion via thiol redox control mechanisms [194]. Pancreatic ductal carcinoma is a malignant solid tumor with poor prognosis. An immunohistochemical analysis by Nakamura et al. [195] revealed an increased expression of Grx1 in 29/32 cases compared to pancreatic cystadenocarcinoma or normal pancreas tissues. Basal cell carcinoma is one of the most common tumors in the Caucasian population. In a screening of 588 genes by differential hybridization of a human cDNA array, differences in the expression levels of 10 genes, including Grx1, were observed in 10 individual basal cell carcinoma specimens and 2 squamous cell carcinoma in comparison to normal skin [196]. Hence, Grx1 may be associated with the high malignant potential of pancreatic ductal carcinoma and basal cell carcinogenesis.

Human Grx2 has been shown to protect HeLa cells from oxidative stress-induced apoptosis; siRNA-mediated silencing of Grx2 dramatically sensitized the cells to doxorubicin and phenylarsine oxide induced cell death [197]. Corroboratively overexpression of both mitochondrial and cytosolic Grx2 decreased the susceptibility of HeLa cells to apoptosis induced by doxorubicin as well as the antimetabolite 2-deoxy-D-glucose [198]. The cells displayed attenuated cytochrome c release and caspase activation induced by both agents. In addition, Grx2 prevented loss of cardiolipin, the phospholipid anchoring cytochrome c to the inner mitochondrial membrane. In our recent investigation of Grx2 transcript variants in human tissues and transformed cell lines, we confirmed and identified two additional isoforms (Grx2b and Grx2c) derived from alternative transcription initiation and splicing [38]. In normal tissue expression of both Grx2b and Grx2c was restricted to testes, but additionally we were able to demonstrate transcripts in various cancer cell lines. A potential role of these isoforms in tumorigenesis and/or progression remains to be established.

### 3.7. Concluding remark

Research on glutaredoxin systems has progressed fast during the last few years, both with respect to the field of redox regulation and

signaling and the emerging ties of glutaredoxins to iron homeostasis and iron-sulfur cluster biogenesis. For the future it will be more important than ever to identify the targets of glutaredoxin action. The definition of the redox-controlled proteome, the redoxome so to say, under various physiological and pathological conditions in various cell types will likely bring new functions to light and bring us closer to clinical applications of these proteins and the redox circuits they control.

## Acknowledgments

The authors wish to thank Karin Beimborn, Gisela Lesch and Lena Ringden for the excellent administrative assistance. The authors gratefully acknowledge the financial support by the Deutsche Forschungsgemeinschaft, the Kempkes Foundation, the Swedish Cancer Society, the Swedish Children Cancer Society, the Swedish Research Council and the K. and A. Wallenberg Foundation.

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