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A residue outside the active site CXXC motif regulates the catalytic efficiency of Glutaredoxin 3

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Abstract

The glutaredoxin (Grx) family of oxidoreductases has a conserved residue at position 8 that varies between Arginine in Grx1 and Lysine in Grx3. It has been proposed that this Arg/Lys change is the main cause for the 35 mV difference in redox potential between the two enzymes. To gain insights into the catalytic machinery of Grx3 and directly evaluate the role of residue 8 in the catalysis of thiol-disulfide exchange by this enzyme, we synthesized the “wild type” enzyme (sGrx3), and four analogues substituting the lysine at position 8 with arginine, ornithine (Orn), citrulline (Cit) and norvaline (Nva). The redox potential and equilibration kinetics with thioredoxin (Trx1) were determined for each enzyme by fluorescence intensity. While minor effects on redox potential were observed, we found that residue 8 had a more marked effect on the catalytic efficiency of this enzyme. Surprisingly, truncation of the functional group resulted in a more efficient enzyme, Lys8Nva, exhibiting rate constants that are an order of magnitude higher than sGrx3 for both forward and reverse reactions. These observations pose the question why would a residue that reduces the rate of enzyme turnover be evolutionarily conserved? The significant changes in the kinetic parameters suggest that this position plays an important role in the thiol-disulfide exchange reaction by affecting the nucleophilic thiolate through electrostatic or hydrogen bonding interactions. Since the reduced Grx has an exposed thiol that could easily be alkylated, either Arg or Lys could act as a gatekeeper that deters unwanted electrophiles from attacking the active site thiolate.

Introduction

Glutaredoxin 3 (Grx3) of *E. coli*, a member of the thiol/disulfide oxidoreductases of the thioredoxin (Trx1) superfamily, consists of 82 residues, including a redox active motif, Cys-Pro-Tyr-Cys (CPYC), typical of the glutaredoxin family.^{1,2,3,4} These enzymes catalyze the thiol-disulfide exchange reaction via reversible oxidation/reduction of their two active-site cysteine residues. The N-terminal cysteine exhibits an unusually low pK_a value ($pK_a \sim 5$ vs. ~ 8 of free cysteine).^{2,5,6,7,8} The pK_a values of the N-terminal thiolate were found to correlate with the redox potential of enzymes in the Trx1 superfamily.^{9,10,11,12} It is

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commonly accepted that Grx3 acts through a disulfide exchange mechanism consisting of a rate-determining intermolecular nucleophilic attack of the thiolate anion in the reduced enzyme on the disulfide substrate, resulting in a mixed-disulfide intermediate (Scheme 1). This mechanism is supported by the observation that kinetic parameters of CXXS analogs are similar to those of the CXXC motif.^{2,8,13} The mixed-disulfide intermediate is subsequently cleaved by an intramolecular attack of the C-terminal cysteine on the sulfur atom of the N-terminal cysteine to produce the oxidized enzyme and the reduced product.

The structure and dynamics of Grx family active sites can vary significantly despite their apparent similarities. For example, Grx1 and Grx3 have ~33% identity and high structural similarity in their active sites.¹⁴ In addition, they share the same active site motif, CPYC, which is involved in a very similar network of hydrogen bonding: the thiolate of Cys11 is hydrogen-bonded to two backbone amides of Tyr13 and Cys14 and to the S-H of Cys14.^{2,15} Nevertheless, Grx1 and Grx3 exhibit significantly different redox potentials (−233 and −198 mV, respectively)¹⁶ and different substrate specificities.^{1,17}

As these two enzymes have an identical active site motif, CPYC, it stands to reason that amino acid residues external to the active site may account for the difference in redox potential. A recent computational study by Foloppe and Nilsson indicated several residues, and the residue at position 8 in particular, which is a conserved residue in the Grx family (Chart 1).^{15,18,19} In Grx1, Arg8 stabilizes the thiolate ion of Cys11 by electrostatic interaction and hydrogen bonding. Similarly, position 8 in Grx3 is occupied by lysine, and in the fully extended conformation the N₈ of Lys8 occupies the same position as C₈ of Arg8 (Scheme 2).¹⁵ The difference in the location and nature of the cation between Arg and Lys was suggested to be responsible for much of the 35 mV difference in the redox potential between these two enzymes.^{15,16,18} We further reasoned that this residue could also affect reaction kinetics by modulating the nucleophilicity of the active site thiolate. While many experimental studies, as well as the computational studies described above, have largely focused on the thermodynamics of these enzymes (redox potential parameters), the question of enzyme kinetics has not received equal attention.

In order to better assess the role of residue 8 on the catalytic efficiency of the glutaredoxins, we designed a series of analogs to systematically vary the electrostatic and hydrogen bonding interactions of this residue. The natural amino acids arginine and lysine are not isosteric with each other, and no other natural amino acids can reach far enough from the backbone to interact with the active site of the enzyme. As a result, we turned to chemical synthesis to utilize non-coded amino acids that are structurally more related to arginine.^{33,34,35} As shown in Scheme 2, ornithine positions a primary amine to be isosteric with arginine, citulline substitutes the guanidinium moiety with a neutral urea functionality and norvaline eliminates the functional group while maintaining the hydrophobic interactions of the linear side chain.²⁰ Due to its moderate size (84 amino acid long), Grx3 is amenable to chemical synthesis and we have previously established synthetic access to this protein to study the role of selenocysteine in oxidoreductases, and we demonstrated that sGrx3 is functionally equivalent to recombinant Grx3.²⁰

Here we report that the Grx3(Lys8Arg) analog exhibits a 10 mV lower redox potential than the sGrx3, supporting the predictions of previous computational studies.¹⁸ We also report that the Grx3(Lys8Nva), which has no side chain functional group at position 8, is an efficient catalyst with rate constants 10-fold higher in both forward and reverse reactions than the sGrx3.

Results and Discussion

The amino acid sequence of the wild-type Grx3 comprises 82 amino acids (Scheme 3A).⁹ We chemically synthesized five proteins, including the “wild-type” enzyme (sGrx3), Grx3(Lys8Arg), Grx3(Lys8Cit), Grx3(Lys8Orn), and Grx3(Lys8Nva), using solid-phase peptide synthesis (SPPS), native chemical ligation (NCL) and alkylation, followed by purification, Ac-m-deprotection and oxidation (Scheme 3B).^{20,21} All proteins were recovered in multimilligram quantities following HPLC purification. In addition to the substitution at position 8 several other modifications were performed: Cys65Tyr, Met43Nle and Ala38Cys(S-CH₂CONH₂).²⁰ All synthetic analogs of Grx3 were folded by dissolving 0.5 mg of each in 50 µL argon-degassed potassium phosphate buffer.

Typically, the redox potentials of oxidoreductases have been determined using end-point analysis at equilibrium with a known redox pair, such as recombinant *E. coli* Trx1 ($E^0 = -270$ mV, Scheme 1).¹⁶ Since Grx does not possess tryptophan (Trp) residues and hence has little fluorescence, we decided to monitor the progress of the equilibration by monitoring the fluorescence of Trx1, which has two Trp residues (Trp28 and Trp31) close to the active site of Trx1.^{22,23} Oxidation of the Cys residues is associated with conformational changes that affect the position of the Trp residues, resulting in decreased fluorescence.^{22,23} The equilibration of equimolar Grx3 analogs (oxidized form) and Trx1 (reduced form) was easily followed by the decrease in the specific Trp fluorescence of Trx1 at 345 nm (excitation at 295 nm) as a function of time, until equilibrium was attained.^{22,23} The relative quantities of reduced and oxidized Trx1 were determined by measuring the fluorescence intensity at equilibrium in comparison with the fully oxidized and fully reduced Trx1 under identical conditions. The data were fit (Figure 1) to the second-order rate equation (using Excel, Microsoft, USA, see Materials and Methods section and SI). The second-order rate constants (k_1 and k_{-1}) as well as the apparent equilibrium constant, $K_{1,-1}$ (Table 1 and Scheme 1) were calculated by fitting the kinetic data. Using the Nernst equation, (Equation 2) the redox potential differences between Trx1 (-270 mV) and each Grx3 analog were also determined (Table 1).

$$K_{1,-1} = \frac{k_1}{k_{-1}} = \frac{[Grx3_{red}][Trx_{ox}]}{[Grx3_{ox}][Trx_{red}]} \quad (1)$$

$$E = E_0 - \frac{RT}{nF} \ln K_{1,-1} \quad (2)$$

The sGrx3 exhibits redox potential and kinetic parameters consistent with literature values of expressed Grx3 (Table 1, entry 1 and 6). In addition, the values using the fluorescence assay are consistent with our previous sGrx3 studies using HPLC integration of reduced and oxidized Trx1.^{16,20} In order to test the hypothesis that electrostatic interactions involving residue 8 significantly modulate the redox potential of glutaredoxins, we performed a Lys8Arg mutation to mimic the active site of Grx1 in the context of Grx3. In principle, this substitution should lower the redox potential of the enzyme from that of the wt-Grx3 (-198 mV) towards that of Grx1 (-233 mV).^{15,16} Indeed, the redox potential was lowered by 10 mV to -208 mV (Table 1, entry 2), suggesting that the Cys11 thiolate anion is better stabilized by Arg8 than by Lys8. This observation is in general agreement with the prediction of Foloppe and Nilsson that Arg8 in Grx1 can hydrogen bond with the Cys11 thiolate 30–40% of the time while Lys8 in Grx3 forms this interaction less than 10% of the time.¹⁵ Thus, although either Arg or Lys can stabilize the thiolate, the Arg8 residue in Grx3 is expected to have greater occupancy in the active site (Figure 2A, B).

Significant changes were also observed with the redox kinetics of the reaction between Grx3(Lys8Arg) and Trx1, exhibiting k_1 and k_{-1} values 5-fold and 2-fold lower than that of the sGrx3, respectively (Table 1, entry 2). Arg8 is expected to interact with the Cys11 thiolate more efficiently than Lys8, as indicated by its lower redox potential (*vide supra*). Therefore, Grx3(Lys8Arg) is expected to better stabilize the reduced state of the enzyme than the oxidized form. Since the Arg8/Cys11 salt bridge must be broken before the Cys11 thiolate can react with the oxidized Trx1 substrate, tighter binding would be consistent with the observation of a lower k_{-1} .

The structure of Grx1 in the oxidized state gives further insight into the role of Arg8. Upon oxidation, glutaredoxins are known to undergo large conformational changes in the active site region involving both side chains and backbone motions.^{24,25,26} In this structure, the disulfide bond is largely shielded from solvent by the backbone of Arg8 and the guanidinium side chain forms a bivalent electrostatic interaction with Asp37. As a result, the Lys8Arg substitution might result in a tighter interaction between residue 8 and the equivalent Asp34 in Grx3 (Figure 2A, B), which is consistent with the lower k_1 of the Grx/Trx1 reaction. Similarly, this interaction in the oxidized state could also affect the observed redox potential.

In contrast to Lys8Arg, the Nva analog is expected to maintain side chain hydrophobic interactions but eliminate electrostatic interactions, leaving the active site open to solvent. The Nva residue has a linear alkyl side chain, corresponding to the alkyl part of the Lys and Arg side chains (Scheme 2, Figure 2E). Interestingly, the Lys8Nva substitution had only a small effect on redox potential, lowering it by 4 mV in comparison with the sGrx3 (−202 mV vs. −198 mV, Table 1, entry 3). Since the Nva side chain is largely solvent exposed, we propose that solvent replaces the cationic head groups of the Lys or Arg side chains in the Grx1/Grx3 wild type structures. This additional solvation would supplement a stable water molecule that has been observed in simulations of the wild type enzyme. This structural water is thought to stabilize the thiolate of Cys11 by hydrogen bonding to the carbonyl amide of Val52 and amino group of Lys8.²⁷ In the oxidized state, the Asp34 side chain would become more solvated since there are no reasonable cationic residues to replace the salt bridge with residue 8. These changes are likely to counteract one another, resulting in a minor overall change in the redox potential.

In contrast to the relatively unchanged redox potential, the kinetic parameters of equilibration show that the absence of a salt bridge with residue 8 results in a significant increase in both k_1 and k_{-1} . Indeed, the Grx3(Lys8Nva) analog has the fastest rate constants in this series with about 10-fold increase in the reaction rate of both forward (k_1) and reverse (k_{-1}) reactions (Scheme 1; Table 1, entry 3), as illustrated by its shorter time to reach equilibrium (3 minutes vs. ~30 min for the sGrx3, Figure 1). This observation is particularly noteworthy in light of previous predictions that mutation of the charged residue (Arg or Lys) at position 8 in Grx3, or the analogous position in closely related enzymes, to hydrophobic residues, such as Ala, Gln and Leu, would diminish the catalytic rate.^{24,27} The increased rate of equilibration with Trx1 is consistent with a more open active site, which would enable interactions with protein substrates. The rate enhancing effect of water molecules within an enzyme active site have already been documented for several relevant cases^{28,29} and spectroscopic studies support the emerging paradigm that intraprotein water molecules are as essential for biological functions as amino acids.^{30,31,32} In addition, both the oxidized and reduced forms of Grx3 have ground state salt bridges that must be broken during the catalytic cycle. Consistent with this interpretation, we propose that Lys8Arg increases the stability of the salt bridges but slows down the turnover of the enzyme while Lys8Nva eliminates the salt bridge but increases the oxidation and reduction kinetics of the enzyme.

Two additional mutants with non-coded amino acids, citrulline (Cit) and ornithine (Orn), were prepared in order to fine-tune the electrostatic interactions at residue 8. Citrulline is an uncharged isostere of arginine (Scheme 2), which makes the Arg/Cit substitution a useful tool to study the importance of electrostatic interactions versus hydrogen bonding in enzymes and other proteins.^{33,34,35} In the context of Grx3, the urea side chain of Cit is expected to adopt a similar conformation to Arg with both NH hydrogen bond donating groups interacting with the thiolate (of Cys11) or carboxylate (of Asp34) anion in the oxidized or reduced state respectively (Figure 2C). While this interaction is expected to be less stabilizing, it should affect the reduced and oxidized states of the enzyme to the same degree, resulting in little change to the redox potential. Consistent with this interpretation, Grx3(Lys8Cit) has a redox potential of -206 mV, similar to -208 mV observed with the Lys8Arg mutant. Considering the equilibration kinetics, it becomes apparent that the weakening of the electrostatic interactions involving residue 8 leads to enhancement of both the forward and back reaction rate constants. Indeed, compared to Lys8Arg, Lys8Cit exhibited a 6-fold increase in both k_1 and k_{-1} . These effects are smaller, but are consistent with the rate enhancements seen in the Nva analog.

The structure of ornithine is intermediate between Lys and Arg. Similar to Lys, ornithine has a terminal primary amine, yet the location of the amine is one methylene closer to the backbone, making it isosteric with the N of the Arg guanidinium group (Scheme 2). As a result, Grx3(Lys8Orn) is expected to have similar conformational properties to Lys8Arg but to provide a more localized N⁺ cation, in contrast to the delocalized bivalent interaction provided by the Arg guanidinium group (Figure 2D). Grx3(Lys8Orn) was found to be similar to the sGrx3 (Lys8) in terms of redox potential, and its reaction rates in both directions are 2-fold smaller than sGrx3 (Table 1, entry 5). These small effects indicate that the exact nature of the positive charge at residue 8 (localized in Lys and Orn vs. delocalized in Arg) has a minor influence on the redox potential and kinetics of this enzyme.

Conclusions

Consistent with predictions from computational studies on the reduced state, the Grx3(Lys8Arg) analog showed a 10 mV lower redox potential than sGrx3. This interaction can account for part of the 35 mV redox potential difference between Grx1 and Grx3. However, somewhat surprisingly, when this residue is replaced by an unnatural amino acid with altered polarity, only minor changes in redox potential were observed.

Oxidoreductases are often characterized primarily by their redox potentials. Nevertheless, since glutaredoxin is an efficient enzyme, the kinetic parameters are important for understanding the role of these proteins in biology. The kinetics of equilibration of Grx3 with Trx1 showed more significant differences between the analogs. Since the Grx3(Lys8Nva) has no side chain functional group at position 8, it cannot directly interact with the active site. Yet, this analog was found to be the most efficient catalyst with rate constants an order of magnitude higher in both forward and reverse reactions as compared with sGrx3. This suggests that breaking electrostatic interactions involving residue 8 in the reduced and the oxidized state contributes approximately 1.4 kcal/mol to the activation barrier for catalysis. Similarly, the comparison between the Grx1-like analog, Lys8Arg, and its neutral urea analog, Lys8Cit, reveals a five-fold increase in both rate constants with little effect on redox potential.

In light of these findings, it is somewhat surprising that a positive charge at position 8 is conserved throughout the glutaredoxin family.⁹ Furthermore, electrostatic interactions between either Arg8 or Lys8 and the Cys thiolate are observed in the NMR structures of both reduced Grx1 and Grx3.^{2,8,9} Why would a residue that reduces the rate of enzyme

turnover be evolutionarily conserved? Although residue 8 is not directly involved in the catalytic mechanism, it affects the nucleophilic thiolate through electrostatic or hydrogen bonding interactions. The significant changes in the kinetic parameters suggest that this position plays an important role in the thiol-disulfide exchange reaction. Since the reduced Grx has an exposed thiol that could easily be alkylated, the Arg/Lys could act as a gatekeeper that deters unwanted electrophilic attacks on the active thiolate. There are several experimental precedents for modulation of an enzyme active site to protect against undesirable side reactions. For example, the aromatic side chain of Tyr270 of glutathione synthetase forms a hydrophobic face against the thiol moiety of glutathione (GSH), which prevents undesirable side-reactions of this reactive thiol.³⁶ Furthermore, the reactive radical intermediates generated in the cobalamin (Vitamin B12) enzymes are protected from side reactions by spatial isolation inside a TIM barrel-like structure.³⁷ Finally, the 4-OT enzyme (and the 4-OT family), which catalyzes the tautomerization of 4-oxalocrotonate, has a conserved N-terminal proline that acts as a general base.³⁸ We have shown previously that the 4-OT(Pro1Ala) mutant catalyzes the reaction but the primary amine of Ala1 residue becomes reactive and attacks the product of the reaction in Michael-type alkylation.³⁹ This is probably the reason for the conservation of an N-terminal proline in these enzymes; to protect the enzyme from Michael-type alkylation by the product of the natural reaction. In this manner, a compromise between catalytic efficiency and functional stability has been achieved to optimize the function of the protein *in vivo*. In this work we have used unnatural amino acids to examine subtle changes in electrostatic and solvation in the active site of Grx3. Studies on a wider range of natural and unnatural Grx3 substrates may shed further light on the role of these enzymes in mediating complex redox pathways in bacterial cells.

Material and Methods

General

Buffers for kinetic measurements were prepared using deionized water (MilliQ). KH_2PO_4 and K_2HPO_4 were purchased from Fisher Biotech. Recombinant *E. coli* Trx1 was purchased from Promega Corp.

Design of Grx3 analogs

Synthetic Grx3 analogs were synthesized as previously described with minor modifications.²⁰ For the ligation site we have selected the bond between Ala37 and Ala38, which lies approximately in the middle of the peptide chain. The solvent exposed residue, Ala38, was substituted by Cys to allow for the native chemical ligation protocol.²¹ Met43 was replaced by norleucine (Nle) to prevent formation of undesired oxidation products during sample handling.^{20,40} Cys65, which has no structural or mechanistic role,² was replaced by Tyr to prevent dimerization side products.

Peptide Synthesis—Peptides were prepared either manually or by machine-assisted solid-phase peptide synthesis (SPPS), typically on a 0.2 mmol scale using the *in situ* neutralization/HCTU activation procedure for Boc-SPPS.⁴¹ The peptide coupling was carried out with 11-fold excess (except for the non-coded amino acids, which were used in 3-fold excess) of activated amino acid for 20 min.

The C-terminal peptide Grx3(Cys38-Lys82) and the five different N-terminal analogs Grx3(Ala1-Ala37) with Ala37 in the form of a thioester derivative (Grx3(1-37)-COSR, Grx3(1-37)(Lys8Arg)-COSR, Grx3(1-37)(Lys8Cit)-COSR, Grx3(1-37)(Lys8Orn)-COSR, Grx3(1-37)(Lys8Nva)-COSR)) were prepared either manually or by machine-assisted SPPS. The active site Cys11 and Cys14 were protected with acetamidomethyl groups (Acm).

The Cys-peptide Grx3(Cys38-Lys82) was synthesized using the Boc-Lys(2ClZ)-OCH₂-Pam resin as described above. Upon completion of the polypeptide assemblies they were deprotected and cleaved from the resin by treatment of the dry peptide-resin (°300–400 mg) with 10–15 mL HF and ~10% anisole for 1 h at 0 °C. The crude peptide products were precipitated and washed with cold anhydrous ether, dissolved in aqueous acetonitrile and immediately purified by reversed-phase HPLC using C18 columns (Phenomenex).

Conformationally assisted ligation—Preparation of all Grx3 analogs via native chemical ligation (Scheme 3B) was carried out under folding conditions.²⁰ The progress of the reaction was followed by analytical HPLC, indicating that the reaction was complete within 4 h, affording the desired protein in high yields (40% recovered). A typical reaction mixture included 8 mg of the thioester-peptide analog (~1.1 equiv) and 8 mg Cys-peptide in 700 µL phosphate buffer (200 mM, pH 7.8, ~3 mM peptide) with 7 µL (1.5% v/v) thiophenol. The ligation was performed at room temperature with periodic vortexing.

Cys38 alkylation with iodoacetamide and active site Acm deprotection—While in principle, the ligation site Cys38 can be reduced to Ala before Acm removal of the Cys active site,^{42,43} we have previously shown that alkylation with iodoacetamide at this position does not perturb the thermodynamic or kinetic parameters of Grx3.²⁰ Upon completion of the ligation reaction, thiophenol was removed by ether extraction and excess iodoacetamide (~500-fold) was added. The Cys38-alkylated product was immediately purified by HPLC. The ligated peptide (2 mg) was dissolved in AcOH (400 µL, 10%) followed by addition of I₂ (2.2 equiv, 5 mM I₂/MeOH) to deprotect the Acm groups from the active site cysteines and subsequent oxidation to form disulfide.⁴⁴ The reaction was complete within 2 h as monitored by electrospray mass spectroscopy (ESI-MS). All oxidized products were immediately purified by HPLC, and characterized by analytical HPLC and ESI-MS and found to be pure and have the expected masses.²⁰

Equilibration kinetics and redox potential determination

All folded Grx3 analogs were prepared by dissolving 0.5 mg of each analog in a separate tube using 200 µL argon-degassed potassium phosphate buffer (100 mM, pH 7.0, 1 mM EDTA). In a separate tube Trx1 (0.5 mg) was dissolved in 200 µL of low pH (to minimize background oxidation) potassium phosphate buffer (5 mM, pH 4.86, 1 mM EDTA). The reduced form of Trx1 was prepared immediately before use by incubation of the protein (~500 µM) in 50 mM dithiothreitol (DTT) at room temperature for 1 h, followed by extensive centrifugation-dialysis (Amicon® Ultra 5000 NMWL, Millipore Corp., Bedford, MA) with degassed potassium phosphate buffer (8 × 2 mL). The concentration of each protein was determined by UV (Genesys6 from Thermo Electron Corp.), using the following $\epsilon_{280\text{ nm}}$ values: Trx1 ($\epsilon_{280\text{ nm}} = 13700\text{ cm}^{-1}\text{M}^{-1}$); all Grx3 analogs exhibit the same $\epsilon_{280\text{ nm}}$ value ($\epsilon_{280\text{ nm}} = 6050\text{ cm}^{-1}\text{M}^{-1}$). The $\epsilon_{280\text{ nm}}$ values were calculated using *Sherpa*_{Lite}^{4.0} for Mac.

Determination of the redox potential was carried out as described by Holmgren.^{22,23} Each of the oxidized Grx3 analogs was equilibrated with equimolar concentration of the reduced Trx1 in argon-degassed phosphate buffer (100 mM K₂HPO₄, pH 7.0, 1 mM EDTA) at 25 °C. The progress of each reaction was monitored in a Fluoromax II fluorometer (Jobin Yvon SPEX Instruments S.A., Inc.) following the decrease in the specific tryptophan fluorescence of Trx1 at 345 nm (excitation at 295 nm). The amounts of reduced and oxidized Trx1 were derived from the equilibrium fluorescence data in comparison with the fluorescence data of the fully reduced and fully oxidized (upon addition of 100-fold excess oxidized glutathione) proteins. The linear background air-oxidation rate was found to be negligibly small under the reaction conditions. The second-order rate constants (k_1 and k_{-1}), as well as the apparent

equilibrium constant, $K_{1,-1}$, were calculated by fitting the kinetic data to the second-order rate equation (SI). Using the Nernst equation, the redox potential differences between Trx1 (−270 mV) and each of the Grx3 analogs were calculated. Our control was the sGrx3 analogue, which exhibits redox potential and kinetic parameters consistent with literature values observed with HPLC separations methods.^{16,20}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.

General mechanism of the redox exchange between Trx1 and Grx3 analogs.

Scheme 2.

Amino acids used for the synthetic mutants of Grx3 (Arg = arginine; Orn = ornithine; Nva = norvaline; Cit = citrulline).

Grx-source	8	11	14
Grx1-Ec	VIFG R SG C PY C VRAKDLA		
Grx3-Ec	EIYT K ET C PY C HRAKALL		
Grx1-Hu	VVFI K PT C PY C RRAQEIL		
Grx2-HuM	VIFS K TS C SY C TMAKKLF		
Grx2-MoM	VIFS K TS C SY C SMAKKIF		
Grx-Le	AVFS K TY C PF C VSVKDLL		
Grx1-Sc	FVAA K TY C PY C KATLSTL		
Grx-Vv	TIFV K YT C PF C RNALDIL		
Grx-Hi	VIFG R PG C PY C VRAKNLA		
Grx-Pig	VVFI K PT C PF C RKTQELL		
Grx-Chick	TLFV K GS C PY C KNAIVLL		

Chart 1.

Sequence alignment of the active site vicinity of various glutaredoxins, taken from ref. 19. Grx1-Ec and Grx3-Ec are Grx-1 and Grx-3 from *E. coli*, respectively; Grx1-Hu and Grx2-HuM are Grx1 and Grx2 from human and human mitochondrial precursor, respectively; Grx2-MoM from mouse mitochondrial precursor; Grx-Le from tomato; Grx-Sc from yeast; Grx-Vv from Vaccinia Virus; Grx-Hi from *H. influenzae*; Grx-Pig from pig; and Grx-Chick from chicken.¹⁹

Scheme 3.

A The amino acid sequence of Grx3 with the two active site residues, Cys11 and Cys14 highlighted in orange, Lys8 in brown, Ala37 and Ala38 in green, Met43 in red, and Cys65 in blue. **B.** General approach to the synthesis of Grx3 and its analogs.²⁰ The two peptides Grx3(1–37)-MPAL and Grx3(C38–82) were ligated in PB (200 mM, pH 7.8, ~3 mM peptides) with the addition of thiophenol, followed by alkylation of Cys38 with iodoacetamide and purification. The product, Grx3(A38X), was deprotected and oxidized in one step by iodine in 10% AcOH. X = (S-CH₂CONH₂)Cys.

Figure 1. Redox equilibration of the different oxidized Grx3 analogs with reduced Trx1, on a relative fluorescence scale ($1-(F_t/F_0)$ as a function of time), during the first 40 minutes of the equilibrations. The best calculated fit is indicated by a different shade of the same color for each curve.

Figure 2. Schematic presentation (based on the NMR structure of Grx3) of the active site (CPYC) hydrogen bonding and electrostatic interactions network in different Grx3 analogs with position 8: A. Lys; B. Arg; C. Cit; D. Orn; E. Nva.^{15,24} Asp34 interactions are indicated as well.⁴⁵

Table 1

Redox potentials and kinetic values obtained from direct protein-protein equilibria between reduced Trx1 and oxidized Grx3 analogs (Fig. 3). Redox potentials were calculated by applying $K_{1,-1}$ to the Nernst equation (Eq. 2). The second-order rate constants (k_1 and k_{-1}), as well as the apparent equilibrium constant, $K_{1,-1}$ (Eq. 1), were calculated by fitting the kinetic data to the second-order rate equation. Data of entries 6–8 were taken from ref. 16, kinetic parameters are not available (na).

Entry	Protein	E^0 (mV)	$K_{1,-1}$	$k_1(\text{M}^{-1}\text{s}^{-1})$	$k_{-1}(\text{M}^{-1}\text{s}^{-1})$
1	sGrx3	-198 ± 2	260 ± 50	1117 ± 160	4.3 ± 0.5
2	Grx3(Lys8Arg)	-208 ± 1	130 ± 15	235 ± 10	1.8 ± 0.2
3	Grx3(Lys8Nva)	-202 ± 2	193 ± 30	9460 ± 400	48.7 ± 7.6
4	Grx3(Lys8Cit)	-206 ± 1	149 ± 10	1425 ± 7	9.6 ± 0.8
5	Grx3(Lys8Orn)	-199 ± 1	253 ± 30	507 ± 20	2.0 ± 0.2
6	wt-Grx3	-198	na	na	na
7	wt-Grx1	-233	na	na	na
8	wt-Trx11	-270	na	na	na