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Direct NMR observation of the Cys-14 thiol proton of reduced *Escherichia coli* glutaredoxin-3 supports the presence of an active site thiol-thiolate hydrogen bond

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Abstract The active site of *Escherichia coli* glutaredoxin-3 (Grx3) consists of two redox active cysteine residues in the sequence -C_{11}-P-Y-C_{14}-H-. The ^{1}H NMR resonance of the cysteine thiol proton of Cys-14 in reduced Grx3 is observed at 7.6 ppm. The large downfield shift and NOEs observed with this thiol proton resonance suggest the presence of a hydrogen bond with the Cys-11 thiolate, which is shown to have an abnormally low pK_a value. A hydrogen bond would also agree with activity data of Grx3 active site mutants. Furthermore, the activity is reduced in a Grx3 H15V mutant, indicating electrostatic contributions to the stabilization of the Cys-11 thiolate.
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Key words: Glutaredoxin; Thiol pK_a; Enzyme mechanism

1. Introduction

Glutaredoxins (Grxs) are members of a family of thiol-disulfide oxidoreductases, including thioredoxin (Trx), protein disulfide isomerase (PDI) and DsbA. These enzymes have a redox active disulfide and a similar architecture, known as the thioredoxin fold [1,2]. Grx1 and Grx3 from *Escherichia coli* are the smallest members (9 kDa) in the thioredoxin superfamily including this fold. All thiol-disulfide oxidoreductases contain the active site sequence -C-X-X-C-, where the N-terminal active site cysteine thiol has a significantly lower pK_a value than a free cysteine thiol (p$K_a \sim 9$). The pK_a value of this thiol has been shown to correlate with the redox potential of the different enzymes [3,4]. The N-terminal active site thiol of Trxs is highly nucleophilic with a thiol pK_a value of ~ 7 [5]. The corresponding active site thiols of DsbA and PDI have pK_a values of 3.2–3.5 [6] and 4.5 [7] respectively, resulting in good leaving group properties for the thiolate anions [8,9]. Like Trx, Grxs are reducing proteins, with low thiol pK_a values for the N-terminal active site cysteine [10–12]. The leaving group ability of the thiolate anion is utilized in a characteristic reaction of Grxs – the reduction of glutathione mixed disulfides (R-SG). In this reaction Grx reduces peptide-SG mixed disulfides with high specificity, and the intermediate Grx-SG mixed disulfide is subsequently reduced by a second glutathione molecule [13–15]. The reduction of the Grx-SG intermediate is facilitated by the favorable leaving group properties of the Grx active site thiolate [16]. Grxs are also reductants for the active site disulfide in ribonucleotide reductase (RR) [17], in which the intermediate mixed disulfide between Grx and RR is released by an intramolecular reaction.

Due to the large impact of the thiol pK_a on the redox potential, considerable effort has been invested to understand the source of stabilization of the thiolate anion. Studies of Trx [18–20], PDI [7] and DsbA [4,21], have shown that the residues between the active site cysteines have a significant influence on the redox potential and thiol pK_a, presumably because of different electrostatic properties of the mutants. The problem is, however, more complex, as the redox potentials of *E. coli* Grx1 and Grx3 differ by 35 mV [22] despite identical active site sequences.

Based on the NMR structure of reduced *E. coli* Trx, a hydrogen bond between the N-terminal active site cysteine thiolate and the C-terminal active site cysteine thiol has been proposed to stabilize the thiolate anion [23]. The NMR structure of a quadruple mutant of reduced human Trx showed no evidence of the corresponding hydrogen bond [24], but instead the thiolate anion was suggested to be hydrogen bonded to the backbone amide of the C-terminal active site cysteine, as in the structure of the oxidized form [24]. A later X-ray structure of human Trx did not support this interpretation of the NMR structure of human Trx but instead demonstrated an active site thiol-thiolate hydrogen bond similar to that found in *E. coli* Trx [25]. Also in a crystal structure of reduced DsbA, a similar thiol-thiolate hydrogen bond has been identified, as well as hydrogen bonds involving the backbone amides of His-32 and Cys-33, and the side chain of His-32 [26].

Here we describe the direct observation of the ^{1}H NMR resonance of the thiol proton of the C-terminal active site cysteine, Cys-14, in reduced *E. coli* Grx3 at 7.6 ppm. The large downfield shift and NOEs observed with this thiol proton resonance suggest the presence of a hydrogen bond with the Cys-11 thiolate.

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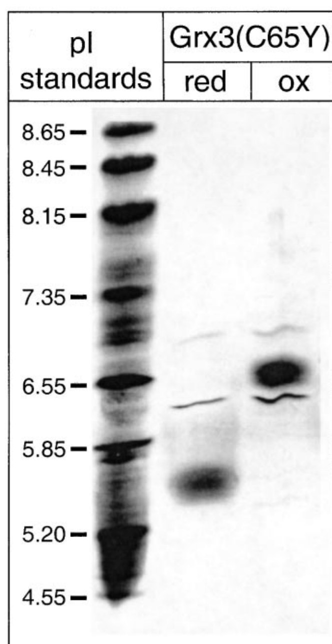

Fig. 1. Isoelectric focusing gel of Grx3 C65Y. Lane 1, pI standard; lane 2, reduced Grx3 C65Y; lane 3, oxidized Grx3 C65Y.

2. Materials and methods

2.1. Growth and purification of Grx3

Grx3 enriched in [$^{13}C^{\beta}$]-L-cysteine was prepared essentially by the method described by Jeng et al. [27]. Briefly, BL-21(DE3) cells containing a pET-Grx3 plasmid were grown at 37°C in 1 l LB medium. Approximately 30 min prior to induction, the medium was supplemented with 50 mg of [$^{13}C^{\beta}$]-L-cysteine and once an OD_{600} of 0.5 was reached, expression was induced with 0.5 mM IPTG (final concentration). The induced cells were harvested after 6 h by centrifugation. The bacterial pellet was dissolved in 6 volumes of 50 mM Tris-Cl, 1 mM EDTA, pH 7.5 and lysed using a combination of lysozyme (0.2 mg/ml) and sonication. The crude extract obtained after centrifugation was heated to 75°C for 5 min, whereafter precipitated protein was sedimented by centrifugation and discarded. The supernatant was dialyzed extensively against 20 mM Tris-Cl, 1 mM EDTA, pH 9.5 and applied to a 150 ml column of DEAE-cellulose (DE-52 Whatman) equilibrated with the same buffer. The column was washed with one volume of 10 mM Tris-Cl, 1 mM EDTA, pH 9.5 and subsequently eluted with 1 l 50 mM Tris-Cl, 1 mM EDTA, pH 8.0. The eluted protein was concentrated by ultrafiltration using a YM-3 membrane (Millipore). The concentrated protein was applied to a column (100 cm × 3 cm^2) of Sephadex G-50 (Pharmacia Biotech Inc.), equilibrated with 100 mM potassium phosphate, 1 mM EDTA, pH 7.0.

Mutants of Grx3 (E9S, C14S, C14A, H15V and C65Y) were constructed by PCR using mutagenic primers (Åslund, F., Spyrou, G. and Holmgren, A., unpublished results). The Grx3 mutants were expressed without the supplement of [$^{13}C^{\beta}$]-L-cysteine. Otherwise, expression and purification followed the protocol described above.

2.2. Enzyme activity assay

The standard coupled assay for glutaredoxin activity, using β-hydroxyethyl disulfide (HED assay) was performed as described by Holmgren [15]. A standard of purified *E. coli* Grx1 was used in each experiment as a positive control. One unit was defined as the oxidation of one μmol of NADPH per min [28]. Specific activity was defined as units per mg enzyme.

2.3. Isoelectric focusing

Native isoelectric focusing of the reduced and oxidized forms of Grx3 C65Y was performed on the Pharmacia PhastSystem using a PhastGel IEF 3-9. The Grx3 C65Y mutant was used in the experiment to avoid dimerization of the oxidized protein. Reduced Grx3 was prepared by pre-incubation with 10 mM DTT.

2.4. NMR data collection

All NMR spectra were recorded at 28°C and a ^{1}H frequency of 600 MHz on a Bruker DMX 600 NMR spectrometer. The COSY and NOESY spectra of wild-type Grx3 were available from a previous study [29]. ^{13}C-HSQC spectra [30] were recorded using Grx3 samples enriched in [$^{13}C^{\beta}$]-cysteine. Each spectrum was recorded with $t_{1max} = 100$ ms, $t_{2max} = 205$ ms and a total recording time of 35 min.

2.5. Determination of pK_a values from NMR data

A total of 19 ^{13}C-HSQC spectra were recorded at different pH values between 5.2–11.0. The pH of each sample was measured before and after recording the NMR spectrum. The pK_a of the cysteine thiols were determined by fitting the data to the Henderson–Hasselbalch equation

$$\delta = \delta_{HA} - \left\{(\delta_{HA} - \delta_{A})/[1 + 10^{(pK_a - pH)}]\right\} \quad (1)$$

where δ is the measured chemical shift of the β-carbon, and δ_{HA} and δ_{A} are the corresponding $^{13}C^{\beta}$ chemical shifts of the protonated and deprotonated side chain respectively. As full transitions were not observed and the number of data points was not sufficient to accurately define the inflection point, only upper or lower limits for the pK_a values could be obtained.

3. Results

3.1. The N-terminal active site cysteine has a pK_a < 5.5

The isoelectric focusing gel (Fig. 1) shows that Grx3 undergoes a shift in pI from 6.5 in the oxidized protein to 5.5 in the reduced form. This shift corresponds to a gain of a net negative charge upon reduction consistent with a fully ionized Cys-11 thiolate in reduced Grx3. A similar shift to higher pI upon oxidation has been noted for rat liver [31] and pig liver [32] Grx. Furthermore, the pH titration of reduced Grx3

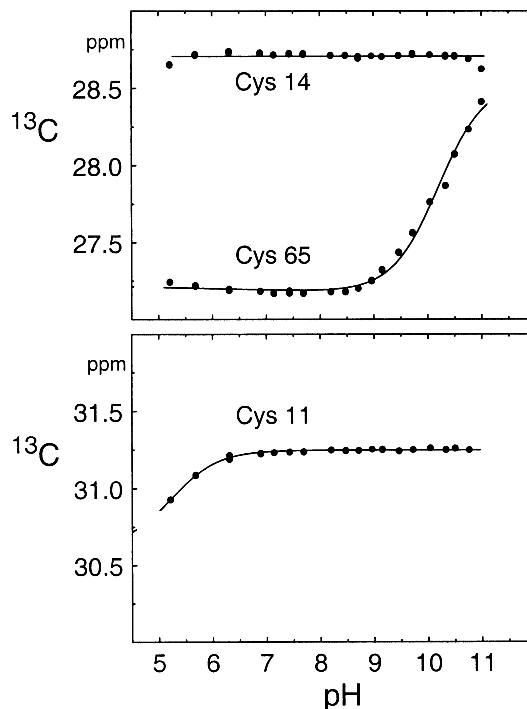

Fig. 2. Cysteine $^{13}C^{\beta}$ chemical shifts in reduced Grx3 vs. pH.

Table 1
Comparison of ^{1}H chemical shifts (ppm) and coupling constants (H_z) for selected residues in the active site of *E. coli* Grx3 in the reduced form (Grx3 red.) at pH 6.8 and in the mixed disulfide complex between Grx3 C14S/C65Y and glutathione (Grx3-SG at pH 6.0); large differences are highlighted

	H^N	H^α	$H^{\beta2}$	$H^{\beta3}$	H^γ	$^3J_{HNH\alpha}$	$^3J_{H\alpha H\beta2}$	$^3J_{H\alpha H\beta3}$	$^3J_{H\beta2H\gamma}$	$^3J_{H\beta3H\gamma}$
Residue 13										
Grx3 red.	**9.43**	**4.37**	3.25	3.02		**8.7**	large[a]	small[a]		
Grx3-SG	**6.61**	**3.63**	3.17	2.89		**4.9**	large[a]	small[a]		
Residue 14										
Grx3 red (Cys)	**9.79**	3.94	**2.63**	**3.60**	**7.61**	6.8	small[a]	large[a]	large[a]	small[a]
Grx3-SG (Ser)	**7.40**	3.97	**3.58**	**4.32**	**5.54**	7.6	small[a]	large[a]	large[a]	small[a]
Residue 15										
Grx3 red.	**8.09**	4.20	3.23[b]	3.23[b]		4.4	–[b]	–[b]		
Grx3-SG	**7.05**	4.17	3.18[b]	3.18[b]		4.5	–[b]	–[b]		

[a]'Large' and 'small' refer to COSY multiplet patterns indicative of trans and gauche conformations of the respective H-N-C-H and H-C-C-H moieties.
[b]Degenerate β-proton resonances.

(Fig. 2) shows a change in ^{13}C chemical shift for Cys-11 C^β below pH 6.5. Since the protein starts to unfold at pH values below 5.5, as judged from the disappearance of high and low field shifted resonances characteristic of the folded protein, the titration of the native protein could no longer be followed. It seems clear, however, that the thiol of Cys-11 has a $pK_a < 5.5$, and is thus largely in the anionic form at physiological pH.

The ^{13}C chemical shift of Cys-14 C^β did not change between pH 5.5 and 10.5 (Fig. 2). As the thiol proton is observed in ^{1}H NMR spectra at pH 6 (see below), the pK_a of the Cys-14 thiol must be >10.5. The third cysteine present in native Grx3, Cys-65 [29], has a $pK_a > 10$ (Fig. 2). As the titration experiments were performed in the presence of DTT to maintain the protein in the reduced form, the thiol pK_a of DTT was measured by the same set of experiments from its ^{13}C chemical shifts (at natural abundance). The value determined for the thiol pK_a of DTT was 9.2, and in agreement with literature data [8].

3.2. Conserved active site conformation in reduced Grx3

NMR spectra of wild-type Grx (WT-Grx) and the double mutant Grx3 C14S-C65Y in a mixed disulfide with glutathione [33] (Grx-SG) show very similar chemical shifts, coupling constants and relative NOE intensities for almost all protons of the active site residues. Active site residues for which larger differences were observed between WT-Grx and Grx3-SG are listed in Table 1. The close similarity of the NMR parameters indicates that the reduction of Grx3-SG results in only minor conformational changes. A comparison of the chemical shift differences between the side chain protons of residue 14 is not meaningful, as this residue is cysteine in WT-Grx3 but serine in Grx3-SG, which is needed to trap the complex. Significant chemical shift changes include the amide protons of Tyr-13, Cys-14 and His-15, as well as Tyr-13 H^α. Of all protons in Grx3-SG, these are the ones closest to the Cys-11 S^γ in the NMR structure and they make no van der Waals contacts with glutathione. The large downfield shifts observed in reduced Grx3 strongly suggest that the

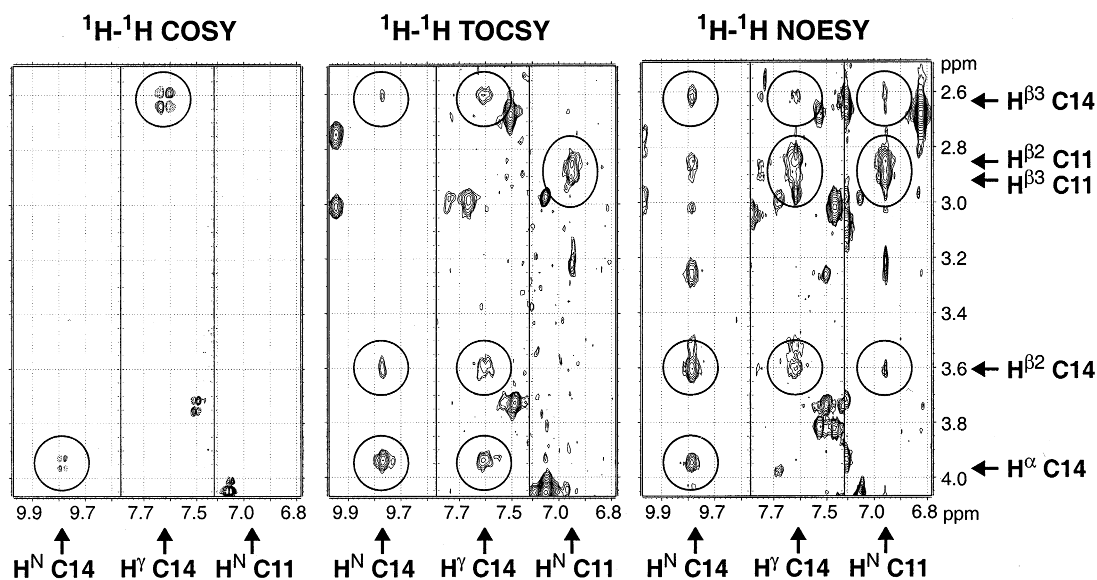

Fig. 3. Slices from COSY, TOCSY (80 ms mixing time) and NOESY (60 ms mixing time) spectra of reduced Grx3, showing cross peaks between Cys-11 and Cys-14.

Table 2
Activity of wild-type and mutants of *E. coli* Grx3

Protein	Specific activity (units/mg)
Grx3	410[a] ± 21
Grx3 C14S	145[a] ± 7
Grx3 C14A	< 10
Grx3 H15V	76 ± 15
Grx3 E9S	408 ± 20

[a][33].

same protons are also near the charged thiolate anion in reduced Grx3. For comparison, the chemical shift change observed for the amide proton of Val-52, which forms a hydrogen bond with glutathione [33] was only 0.69 ppm.

A remarkable chemical shift of 7.6 ppm was found for the Cys-14 thiol proton, H^{γ} (Fig. 3). To date, no thiol protons have been reported for the thiol-disulfide oxidoreductases and they are quite generally rarely observed in NMR studies of proteins and peptides. Among the 174033 chemical shifts currently deposited in the BioMagResBank (January 1999), a total of five thiol proton chemical shifts are reported, which are in the range 1.90–5.05 ppm. A random coil chemical shift of approximately 1.9 ppm at 25°C has been reported for cysteine in aqueous solution [34]. The large downfield shift of Cys-14 H^{γ} is indicative of hydrogen bonding. The thiolate anion of Cys-11 is the most likely hydrogen bond acceptor, since Cys-14 H^{γ} makes stronger NOEs with the β-protons of Cys-11 than those of Cys-14 (Fig. 3).

3.3. C14S, C14A and H15V Grx3 mutants have reduced activity

To test the putative stabilizing effect of the Cys-14 thiol on the Cys-11 thiolate, two mutants, Grx3 C14S and Grx3 C14A, were tested for their activity in the Grx activity assay (HED assay, Table 2). The conservative mutation to serine resulted in a 65% reduction of activity [31]. The mutation of Cys-14 to Ala resulted in a complete loss of activity. To assess the importance of electrostatic interactions with residues in the vicinity of the active site, two additional mutants, Grx3 H15V and Grx3 E9S were tested by the activity assay. Of the residues with charged side chains, His-15 is closest to the active site, whereas the side chain of Glu-9 is rotated away from the active site in the Grx3-SG structure. Conceivably, a positive charge on His-15 could stabilize the Cys-11 thiolate. The activity of the Grx3 H15V mutant was 19% of that of WT-Grx, suggesting that the side chain of His-15 indeed contributes to the stabilization of the Cys-11 thiolate. In contrast, the Grx3 E9S mutant was found to be equally active as the wild-type protein. This demonstrates that the stabilization of the thiolate depends more strongly on the charges near the active site rather than the overall charge of the protein.

4. Discussion

NMR spectra of reduced wild-type Grx3 contain the H^{γ} proton resonance of Cys-14 even when the water resonance is suppressed by selective pre-irradiation. This indicates that the H^{γ} proton is protected from rapid exchange with bulk water, by hydrogen bonding or because of restricted solvent accessibility due to burial in the interior of the protein. The unusually large downfield shift of this proton resonance can be explained by a hydrogen bonding interaction with the negatively charged Cys-11 thiolate anion. Significant downfield shifts were also observed for the H^{N} resonances of Tyr-13 and Cys-14 in reduced Grx3 compared to their chemical shifts in Grx3-SG. Both amide groups are potential hydrogen bond donors for the active site thiolate. Multiple hydrogen bonds stabilizing the thiolate would be analogous to the situation found in reduced DsbA [26]. Hydrogen bonding with the thiol of Cys-14 seems to be an important factor for the low pK_a value of the Cys-11 thiol in Grx3. This conclusion is corroborated by the fact that Grx3 C14S and Grx3 C14A mutants displayed little and no activity, respectively, in the HED assay. The rate limiting step in this assay is thought to be the reduction of a Grx-SG intermediate by glutathione [16], i.e. increased leaving group ability due to stabilization of the thiolate anion should be reflected by enhanced activity in this assay.

In addition to hydrogen bonding, longer range electrostatic effects can also contribute to the stabilization of the thiolate anion in Grx3. Thus, a Grx3 H15V mutant displayed decreased reactivity in the activity assay, indicating a stabilizing interaction with the histidine side chain. In pig liver Grx an arginine side chain is found at the corresponding position of His-15 in Grx3, and the corresponding mutation, R26V, was shown to reduce the activity by 68% [10]. Quite generally, a positively charged residue following the -C-P-Y-C- sequence seems to stabilize the active site thiolate, enhancing Grx activity [35].

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