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Exploring the active site of plant glutaredoxin by site-directed mutagenesis

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Abstract Six mutants (Y26A, C27S, Y29F, Y29P, C30S and Y26W/Y29P) have been engineered in order to explore the active site of poplar glutaredoxin (Grx) ($Y_{26}CPYC_{30}$). The cysteinic mutants indicate that Cys 27 is the primary nucleophile. Phe is a good substitute for Tyr 29, but the Y29P mutant was inactive. The Y26A mutation caused a moderate loss of activity. The YCPPC and WCPPC mutations did not improve the reactivity of Grx with the chloroplastic NADP-malate dehydrogenase, a well known target of thioredoxins (Trxs). The results are discussed in relation with the known biochemical properties of Grx and Trx.

Key words: Glutaredoxin; Thioredoxin; Redox regulation; Dithiol/disulfide exchange

1. Introduction

Thioredoxin (Trx) and glutaredoxin (Grx) are structurally related proteins, the major function of which is to reduce disulfide bridges on other proteins. Both proteins differ by their mode of reduction and their active site sequence (YCP[Y/F]C for Grx and WC[G/P]PC for Trx) [1].

Trxs are fairly well characterized in plants, and it has been shown that there are multiple nuclear genes (close to 20 in the simple model *Arabidopsis thaliana* that encode cytosolic, mitochondrial and chloroplastic isoforms) [2]. In the cytosol and probably mitochondria, Trxs are reduced via NADPH and a flavoprotein, NADPH Trx reductase. Chloroplastic Trxs are reduced in the light by a cascade that involves the photosystem I, [2Fe2S] stromal ferredoxin and an iron–sulfur enzyme, ferredoxin–Trx reductase [3]. The targets of chloroplastic Trxs are well characterized, notably the redox-regulated fructose-1,6-bisphosphatase and NADP-malate dehydrogenase (MDH) [4,5]. Besides the two catalytic cysteines, the active site of Trx involves the conserved tryptophan residue adjacent to the most N-terminal cysteine and a buried aspartate residue that is conserved in all Trx sequences from bacteria to mammalian systems [6–8]. The redox potentials of Trxs are close to −300 mV, a value that makes them very efficient reductants of disulfide bridges [9]. The three-dimensional structure of Trxs is also very well described, all isoforms have a similar fold with a central pleated β sheet surrounded by α helices. The attacking cysteine as well as the active site tryptophan are surface-exposed while the other cysteine is more buried [10].

Grxs are rather well known in bacteria (such as *Escherichia coli*), yeast and mammalian systems. In *E. coli* there are three Grx genes, at least five in yeast and apparently only two in human [11–14]. In all known organisms, Grxs are reduced via a cascade that involves NADPH, glutathione reductase (GR) and the tripeptide glutathione [1]. The redox potential of Grx is estimated to be around −230 mV [15]. Although the targets of Grx are not as well characterized as those of Trx, it is quite clear that Grx is the preferred donor to ribonucleotide reductase and as efficient as Trx with PAPS reductase [16,17]. On the other hand, the information about plant Grxs is more scarce. The questioning of GenBank indicates that there are also multiple genes in plants with rather large variations at the active site (from the canonic YCPYC to the less frequent YCPFC, but also to more exotic forms as GCCMS where the active site sequence is hardly recognizable). Plant Grxs have been purified from several sources (rice and spinach mostly) [18,19]. The protein was found to possess activity in the 2-hydroxyethyl disulfide (HED) reduction assay and also in the more physiological dehydroascorbate (DHA) reduction [18]. Quite recently, Grx has been cloned and overproduced from poplar [20]. It has been shown to be very effective in the DHA reduction, but also to be a good electron donor to a new cytosolic peroxiredoxin (Prx) [21]. Quite interestingly, Trx, Grx and Prx have been found in sieve tubes and the phloem sap, sometimes in very large amounts, a property that suggests a role in the long distance transmission of the redox signal in plants [21–23]. One interesting feature of the poplar Grx is that it is elongated both on the N- and C-termini compared to the mammalian or bacterial Grxs characterized so far. It is thus of interest to investigate the biochemical reactivity of this type of protein in order to compare those data with the ones obtained with shorter versions of this protein in distant organisms. We describe in this paper a series of mutations that help understand the importance of the amino acids of the active site.

2. Materials and methods

2.1. Materials

Purified oligonucleotides, restriction enzymes, DNA polymerase and ligase were either from Eurogentec or from Invitrogen. IPTG,

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Abbreviations: DHA, dehydroascorbate; DTT, dithiothreitol; Grx, glutaredoxin; GR, glutathione reductase; HED, 2-hydroxyethyldisulfide; NADP-MDH, NADP-malate dehydrogenase; Prx, peroxiredoxin; Trx, thioredoxin

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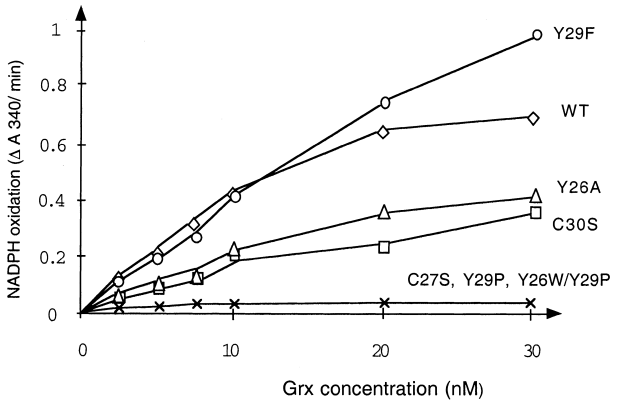

Fig. 1. Reduction of HED by WT and mutated poplar Grxs. All activities were measured at 30°C. After 1 min incubation of the system, the reaction was initiated by addition of Grx.

NADPH, HED, DHA, reduced glutathione (GSH) and GR were from Eurogentec, Boehringer, and Sigma. Chromatographic matrices were from Pharmacia. Kinetic measurements were followed using a Cary 50 spectrophotometer.

2.2. Plasmids and strains

The plasmid used for cloning and expression was pET-3d which carries the resistance for ampicillin. When needed, an additional plasmid (pSBET, carrying a kanamycin resistance) [24] was used to co-transform the expression strain *E. coli* BL21(DE3). The cloning strain was *E. coli* DH5α.

2.3. Mutagenesis

Mutagenesis of the cloned cDNAs was effected by PCR. A strategy similar to the one in [25] was employed, generating two overlapping mutated fragments and in a second PCR reaction the full length mutated sequence. The two 'cloning' oligonucleotides had the following sequences (*Nco*I and *Bam*HI restriction sites underlined): forward 5′-GGGCCATGGCTGGCAGCCCTGAAGCT-3′; reverse 5′-GGGGGATCCTCATTCTAGTTTAAAGTCATC-3′.

The mutagenic oligonucleotides had the following sequences (mutagenic bases in bold): Y_{26}A FOR 5′-ATCTTCTCCAAGTCT**GC**TTGCCCGTATTGT-3′; Y_{26}A REV 5′-ACAATACGGGCAA**GC**AGACTTGGAGAAGAT-3′; Y_{26}W–Y_{29}P FOR 5′-TCCAAGTCT**TGG**TGCCCG**CC**TTGT-3′; Y_{26}W–Y_{29}P REV 5′-ACAA**GG**CGGGCA**CC**AAGACTTGGA-3′; C_{27}S FOR 5′-TTCTCCAAGTCTTA**A**GCCCGTATTGTAAGAAG-3′; C_{27}S REV 5′-CTTCTTACAATACGGGC**T**ATAAGACTTGGAGAA-3′; Y_{29}P FOR 5′-TCTTATTGCCCG**CC**TTGTAAGAAGGCT-3′; Y_{29}P REV 5′-AGCCTTCTTACAA**GG**CGGGCAATAAGA-3′; Y_{29}F FOR 5′-TATTGCCCGT**T**TTGTAAGAGG-3′; Y_{29}F REV 5′-CCTCTTACAA**A**ACGGGCAATA-3′; C_{30}S FOR 5′-TATTGCCCGTATT**C**TAAGAAGGCTAAA-3′; C_{30}S REV 5′-TTTAGCCTTCTTA**G**AATACGGGCAATA-3′.

The template used was the construction pET-Grx3 [20]. After digestion with *Nco*I and *Bam*HI, the full length mutated fragments were cloned into pET-3d. The mutations were verified by DNA sequencing and the recombinant plasmids were used to transform the expression strain and then ampicillin and kanamycin resistant clones selected.

2.4. Expression and purification of the recombinant proteins

The transformed *E. coli* cells were successively multiplied to a final volume of ca. 5 l at 37°C. 100 μM IPTG was added in the exponential phase and the bacteria harvested by centrifugation for 15 min at 5000×*g*. The cells were resuspended in a TE buffer (Tris–HCl 30 mM, pH 8.0, EDTA 1 mM) which also contained 14 mM β-mercaptoethanol for the cysteinic mutants. All subsequent chromatographic steps were effected in the same buffer. The recombinant proteins were purified by ammonium sulfate fractionation (50–90%), Sephadex G50 gel filtration and DEAE Sephacel chromatography. The samples were then concentrated and dialyzed by ultrafiltration in an Amicon cell (Millipore) equipped with a YM 10 membrane under nitrogen pressure. The proteins were stored by aliquots, frozen at −20°C at concentrations of ca. 4 mg/ml. The yield was around 10 mg homogeneous protein per liter culture.

2.5. Biochemical assays

The assays describing the reductions of HED and DHA were performed as described in [20]. The Prx and NADP-MDH assays were as in [21,26]. The preparations of recombinant Grx, Prx and NADP-MDH have been described in [20,21,26].

The Prx reaction was effected in 500 μl cuvettes in the presence of 50 mM K-phosphate buffer, 150 μM NADPH, 1 mM GSH, 0.5 U GR, and 2.5 μM poplar Prx and Grx. The reaction was started by adding 100 μM H_2O_2 after 1 min of incubation at 30°C to permit the reduction of the system. Activity was measured by following the oxidation of NADPH at 340 nm.

The activation medium for NADP-MDH (30 μl) had the following composition: 50 mM Tris–HCl pH 8.0, 5 mM dithiothreitol (DTT), 0.8 μM recombinant sorghum NADP-MDH and 20 μM Grx or Trx as indicated. After 20 min incubation at 20°C, an aliquot of 20 μl was used to determine the activity at 30°C as described in [5].

The purity of the protein preparations was estimated by SDS–PAGE as described by Laemmli [27].

3. Results

3.1. Efficiency of the mutated Grxs in the 'classical' HED and DHA reduction tests

Fig. 1 shows the reactivity of the various mutants in the reduction of the non-physiological substrate, HED. Replacing Tyr 29 by a Phe residue had little effect on the activity, the protein behaving essentially as the wild-type (WT) enzyme. On the other hand, introducing a non-aromatic amino acid instead of either of the Tyr of the active site depressed the Grx activity (Y26A had a catalytic efficiency half the WT and the replacement of Tyr 29 by Pro resulted in an inactive protein). The C30S mutant retained 30% of the activity, but the C27S protein was inactive.

Essentially similar results were obtained in the DHA reduction (Fig. 2). When Tyr 29 was replaced by Pro, the protein was inactive and the replacement of Cys 27 by a serine likewise produced an inactive catalyst. Replacing Tyr 26 by an Ala decreased the catalytic efficiency (ca. 20%). On the other hand, the Y29F and C30S mutations produced better catalysts. It is especially remarkable that the monocysteinic mutant C30S which contains only Cys 27 is more efficient than the WT protein (the activity is nearly doubled at every concentration tested).

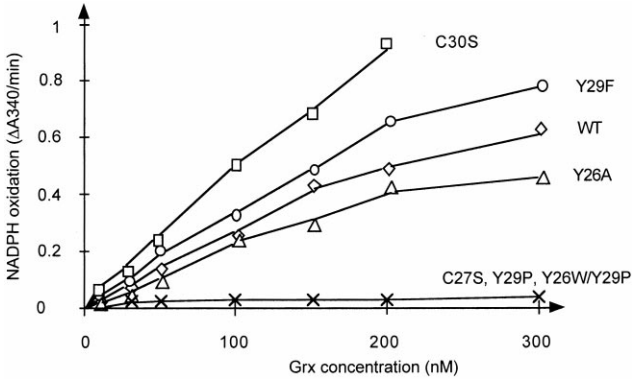

Fig. 2. Reduction of DHA by WT and mutated poplar Grxs. All activities were measured at 30°C. After 1 min incubation of the system, the reaction was initiated by addition of Grx.

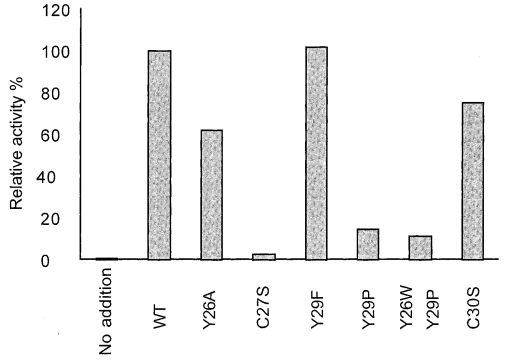

Fig. 3. Activity of Prx with WT and mutated Grxs. The activity is expressed in % of the WT protein. 100% activity is 0.55 ΔOD/min. Details of the reaction are given in Section 2.

3.2. Grx efficiency vs plant type C Prx

It has been demonstrated recently that type C Prx of plants uses both Trx and Grx as proton donors [21]. It was thus of interest to determine if the various mutants generated here are able to sustain the catalytic activity of Prx in this newly described reaction. Fig. 3 shows the results of those experiments. The WT and Y29F mutant show similar activity. On the other hand, replacing the two Tyr residues by non-aromatic side chains decreased the catalytic efficiency. Finally, the C30S mutant retains most of its catalytic efficiency and the C27S protein is inactive.

3.3. Activation of NADP-MDH by mutated Grxs

Two of the mutations that we have engineered in this work transform the Grx active site into a Trx-like active site (YCPPC and WCPPC). It was expected that these mutations should increase the reactivity of Grx vs the NADP-MDH, a well characterized target enzyme of Trx. Fig. 4 shows the reactivity of the various mutants with NADP-MDH. It is interesting that the WT Grx is able to activate the enzyme in the presence of DTT with reasonably good efficiency (nearly half the capacity of the non-physiological Trx *h* at the same concentration of 20 μM). All mutants tested showed catalytic activity, but all mutations decreased the efficiency except for Y26A. The two mutations that were intended to mimic the Trx active site (Y29P and Y26W/Y29P) did not improve the reactivity at all. Interestingly, both the C27S and C30S mutants retained the capacity to activate the NADP-MDH but their efficiencies were strongly reduced (to ca. 25% of the WT).

4. Discussion

4.1. Mutations of the active site cysteines

In the Grx dependent reactions (HED and DHA reduction and Prx activation), the mutation of Cys 27 leads to a virtually inactive enzyme. On the other hand, the mutation of Cys 30 into Ser had either a strongly negative effect (HED), a mildly negative effect (Prx) or a positive effect (DHA). All these data strongly suggest that Cys 27 is the primary nucleophile of the reaction and that Cys 30 is the backup cysteine. Similar results have been obtained with the *E. coli* and human Grx or all Trxs where the catalytic cysteine is always located on the N-terminus side [28–30]. It seems thus that the N- and C-termini extensions of plant Grx do not alter that property. Additionally, these site-directed mutagenesis experiments indicate that Grx can be quite efficient as a single cysteine catalyst, confirming that it can act in the so-called monothiol pathway [31]. Moreover, these experiments suggest that the numerous natural monocysteinic versions of Grx that exist in the databanks are almost certainly functional catalysts.

4.2. Importance of the aromatic residues of the active site

The replacement of the Tyr residue present between the two catalytic cysteines by a proline leads to a drastic decline in reactivity. It is clearly essential to keep an aromatic residue in this position as the Y29F mutant is a very good catalyst in all Grx dependent reactions tested. This Tyr residue has been implicated as one of the ligands necessary for the fixation of glutathione to *E. coli* Grx3 or T_4 Grx [11,32]. On the other hand, it is not necessary to have an aromatic residue in position 26 (N-terminus to the catalytic cysteine) as the Y26A mutant kept at least half of the reactivity of the WT protein. This is very much in contrast with the case of Trx where the removal of the Trp adjacent to the catalytic cysteine has a very strong negative effect [6,33].

4.3. The NADP-MDH is activated by plant Grx and its mutants

The NADP-MDH has been recognized as a target of Trxs since a long time. The chloroplastic Trx *f* is the most efficient, followed by chloroplastic Trx *m*, and the cytosolic Trx *h* [34]. Mammalian Trxs are very poor reductants/activators of this enzyme [35]. All Trxs which have been demonstrated as capable of activating this enzyme have either a WCGPC or a WCPPC active site. By making the Y29P and Y26W/Y29P mutations, we have engineered proteins with active sites that have been transformed into YCPPC and WCPPC. It was thus expected that these mutations could result in Grxs with better reactivity vs the NADP-MDH. The results in Fig. 4 indicate clearly that this is not the case as only the Y26A mutant has an activity comparable to the WT protein and all other mutations decrease the reactivity. It is surprising to observe that WT Grx is able to induce the activation of the enzyme in the presence of DTT (see Fig. 4). This activation is dependent on the concentration of added Grx and on DTT (data not shown). The WT poplar Grx is not as efficient as the cytosolic Trx *h* but more efficient than the human Trx or the Trxs of *Dictyostelium discoideum* [35,36]. We have checked that this reactivity is not due to contaminating *E. coli* Trx in the recombinant Grx preparations. We have indeed quantitated the

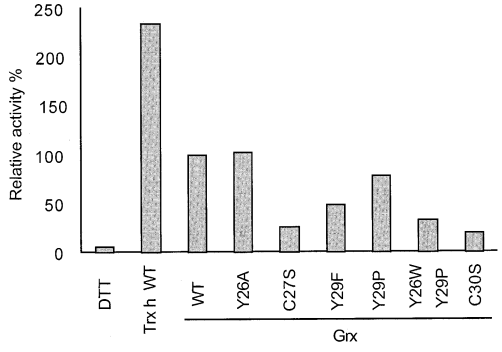

Fig. 4. Activity of NADP-MDH with WT and mutated Grxs. Activities are expressed in % of the WT Grx. 100% activity is 0.4ΔOD/min. Experimental details are given in Section 2.

contamination of those preparations by the NTR/DTNB system and found that it could not exceed 0.3%. At a Grx concentration of 20 μM in Fig. 4, the *E. coli* Trx concentration is thus lower than 60 nM, a value at which no detectable NADP-MDH activation occurs [37]. This activation of NADP-MDH by Grx is likely to be non-physiological since GSH could not replace DTT, even in the presence of NADPH and GR (data not shown). Three additional observations support the proposal that Grx is indeed not a physiological activator of NADP-MDH. First, it seems that there are no chloroplastic sequences for Grx in protein databases. Second, the redox potential of Grx (normally around −230 mV) is not adequate with respect to the ones of the NADP-MDH disulfide bridges (−280 and −300 mV) for an efficient reaction to take place [38]. Third, the fact that the C30S mutant keeps a level of activity similar to the C27S mutant suggests that the role of Grx in this reaction is non-catalytic. We propose thus that Grx acts here in a non-catalytic way to modify the accessibility of the active site to DTT which has the right redox potential. Such a structural role has already been observed in the case of the interaction between the T_7 DNA polymerase and the *E. coli* Trx [39]. The results obtained with the C27S mutant seem to support such a hypothesis since Cys 30 is widely recognized as the non-catalytic one, a property also verified in the other reactions described here.

4.4. Concluding remarks

Mutations similar to those described here have already been performed either on *E. coli* Trx to transform it into a Grx or a DsbA protein [40,41], or on DsbA to transform it into a Grx or a Trx [42]. In most of these mutants, essentially physicochemical characteristics as pK_a values and redox potentials have been determined but few kinetic experiments have been performed except in [40] where the creation of a CGHC site has been shown to be accompanied by an increase in PDI-like activity. In this study, nearly all the mutants that we have generated are less active than the WT protein, in contrast to initial expections. Transforming the Grx active site into a Trx-like active site did not improve the reactivity with NADP-MDH or Trx reductase (data not shown). Similarly to what is described here, simulations of Trx or Grx-like active sites in tryparedoxin 2 did not result in Trx or Grx-like activities [43]. Overall, these data strongly suggest that the structural determinants for Trx or Grx reactivity go beyond the sequence of the active site.

Nevertheless, the series of mutants generated here help to understand the functioning of plant Grxs. The catalytic cysteine as well as the importance of an aromatic residue inside the regulatory sequence and adjacent to the backup cysteine have been uncovered. An additional interesting finding of this study is the capacity of Grx to activate the NADP-MDH. This enzyme has always been reputed as promiscuous as it accepts many different Trxs as regulators, in contrast to fructose-1,6-biphosphatase that requires selectively Trx *f*. The data presented here indicate that the DTT NADP-MDH activation test should be used cautiously, as Grx is also active in this process despite the fact that it probably does not act in a catalytic way.

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