FEBS 27883 FEBS Letters 555 (2003) 443–448

Evidence for a subgroup of thioredoxin *h* that requires GSH/Grx for its reduction

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Received 6 October 2003; revised 3 November 2003; accepted 3 November 2003

First published online 18 November 2003

Edited by Ulf-Ingo Flügge

Abstract Poplar thioredoxin *h*4 (popTrx*h*4) and a related CXXS type (popCXXS3) are both members of a plant thioredoxin *h* subgroup. PopTrx*h*4 exhibits the usual catalytic site WCGPC, whereas popCXXS3 harbors the non-typical active site WCMPS. Recombinant popTrx*h*4 and popCXXS3 are not reduced either by *Arabidopsis thaliana* NADPH-dependent thioredoxin reductases (NTR) A and B or by *Escherichia coli* NTR. We report here evidence that a poplar glutaredoxin as well as three *E. coli* Grxs are able to reduce popTrx*h*4. PopTrx*h*4 is able to reduce several thioredoxin targets as peroxiredoxins or methionine sulfoxide reductases. On the other hand, popCXXS3 exhibits an activity in the presence of glutathione and hydroxyethyldisulfide. Except for examples of glutathiolation, these are the first two examples of a direct interconnection between the thioredoxin and glutathione/glutaredoxin systems.

Key words: Thioredoxin *h*; Glutaredoxin; Thiol reduction; *Populus*

1. Introduction

Two major systems maintain the thiols essentially in the reduced state in the cytosol, the thioredoxin (Trx) system and the glutathione/glutaredoxin system (GSH/Grx). In contrast to non-photosynthetic organisms, plants exhibit a very complex Trx system since at least 21 genes encoding Trxs have been identified in the *Arabidopsis thaliana* sequenced genome [1]. One of the characteristics of plants is that they contain a complex chloroplastic Trx system, with different Trx types (Trx *m*, *f*, *x* and *y*). The reduction of these Trxs is mediated by a heterodimeric ferredoxin-dependent Trx reductase (for review see [2,3]). Recently, a mitochondrial system has been described in *A. thaliana*, involving a Trx *o* that is reduced by a NADPH-dependent Trx reductase (called NTRA in *A. thaliana*) [4]. Plants also exhibit another Trx type, called *h* for heterotrophic. The Trxs *h* (at least eight genes identified in *A. thaliana*) are divided into three subgroups [1]. The reduction of already described Trxs *h* is mediated by a cytosolic NTR (called NTRB in *A. thaliana*) homologous to the mitochondrial one. Two additional related genes encoding Trx *h*-like proteins have also been detected in the *A. thaliana* genome [1]. Both exhibit a modified active site, WC[I/L]PS and have been named atCXXS. Until now, the biochemical properties and the physiological role of proteins of this subgroup remain largely unknown, except for a partial study in [5].

Besides the Trx system, the GSH/Grx system is also involved in the regulation of the thiol redox state of the cells. As for Trxs, plants exhibit a very complex GSH/Grx system since at least 31 genes encoding Grx have been identified in the *A. thaliana* genome. Grx is reduced by glutathione, transmission of the redox signal being mediated from NADPH to glutathione via glutathione reductase, a flavoprotein.

We report here the biochemical characterization of two poplar proteins, members of the third Trx *h* subgroup, called popTrx*h*4 and popCXXS3. Both proteins are not reduced by either atNTRA or B. We show that a poplar Grx is able to reduce popTrx*h*4 and that popCXXS3 exhibits a hydroxyethyldisulfide (HED) reducing activity in the presence of GSH, providing the first examples of a direct relationship between the Trx and GSH/Grx systems in plants.

2. Materials and methods

2.1. Materials

NADPH was obtained from Roche Molecular Biochemicals; L-MetSO, glutathione reductase from yeast, and GSH were from Sigma. Dithiothreitol (DTT), isopropyl-1-thio-β-D-galactopyranoside, kanamycin, and ampicillin were from Fermentas. HED was from Acros Organics.

2.2. Cloning of popTrxh4 and popCXXS3

Two sets of oligonucleotides primers were constructed based on the available expressed sequence tags (ESTs) for popTrx*h*4 and popCXXS3. The upstream oligonucleotides were synthesized homologous to the coding strand, including an addition of four C and of a *Nco*I restriction site (underlined) at the 5′-end with the following sequences: popTrx*h*4: 5′-CCCCCCATGGGACTTTGCTTGGATAAGCAT-3′; popCXXS3: 5′-CCCCCCATGGCTGAGAGCCAAGAACAGCAACCT-3′.

The downstream oligonucleotides were complementary to the coding strand, featuring at the 5′-end four additional C and a *Bam*HI restriction site (underlined) with the following sequences: popTrx*h*4: 5′-CCCCGGATCCTCATTTGTCACTAGGGGGCAA-3′. popCXXS3: 5′-CCCCGGATCCCTATGCATTATACGCACGAAT-3′.

The PCR reactions were performed on leaf library and the amplified fragments with the expected size were purified, digested with the appropriate enzymes and cloned into the pET-3d expression plasmid as described previously [6] in order to obtain the recombinant plasmids.

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Abbreviations: DTNB, 5-5′ dithiobis nitrobenzoic acid; DTT, dithiothreitol; Trx, thioredoxin; Grx, glutaredoxin; Prx, peroxiredoxin; MsrA, methionine sulfoxide reductase A; HED, hydroxyethyldisulfide

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doi:10.1016/S0014-5793(03)01301-2

2.3. Expression and purification of the recombinant proteins

The procedures for the expression and purification of *A. thaliana* NTRB, poplar Trx*h*1, *h*2, *h*3 and WT and mutant Grxs and poplar type II peroxiredoxin (PrxII) are described elsewhere [7–11]. *A. thaliana* NTR A and *Escherichia coli* Grxs were obtained from Dr. Y. Meyer and Dr. A. Vlamis-Gardikas respectively. The procedures for the expression and purification of poplar PrxQ (popPrxQ) and methionine sulfoxide reductase (MsrA) will be described elsewhere.

The recombinant plasmids were used to transform *E. coli* strain BL21(DE3), which was also cotransformed with the helper plasmid pSBET as described previously [12]. After culture harvesting and cell suspension sonication, the resulting sample was fractionated by ammonium sulfate precipitation. The fraction precipitating between 40 and 80% of the saturation was submitted to chromatographic steps consisting of a G50 gel filtration column followed by a DEAE-Sephacel ion exchange. The final yield was nearly 20 mg protein l^{-1} culture for the two preparations. The proteins were stored frozen at −20°C in Tris–HCl 30 mM pH 8.0 and EDTA 1 mM.

2.4. Activity measurement

2.4.1. NTR reduction. The reduction of Trx by NADPH and recombinant NTR from either *E. coli* or *A. thaliana* was followed at 412 nm using 5-5′ dithiobis nitrobenzoic acid (DTNB) as a substrate [7].

2.4.2. PopPrxQ activity. The reduction of H_2O_2 by popPrxQ in the presence of either different popTrx*h*s or Grxs was followed spectrophotometrically using a Cary 50 spectrophotometer. When using the NTR system, a 500 μl cuvette contained the following components: 30 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 μM NADPH, 500 μM H_2O_2, 0.8 μM atNTRB, 16 μM popTrx*h* and 2 μM popPrxQ. Using the GSH/Grx system, the reaction mixture (500 μl) contained 30 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 μM NADPH, 1 mM GSH, 0.5 IU glutathione reductase, 6 μM Grx, 16 μM popTrx*h*4, 500 μM H_2O_2 and 2 μM popPrxQ.

2.4.3. PrxII activity. The interaction between popTrxs and popPrxII was analyzed by measuring the disappearance of H_2O_2. The reaction mixture (100 μl) contained 30 mM Tris–HCl pH 7.0, 500 μM DTT, 4 μM popPrxII and 36 μM popTrx*h*4. The reaction was started by adding 500 μM H_2O_2. At several incubation times, 5 μl was mixed with 495 μl of FOX1 (ferrous oxidation in xylenol orange) reagent [13]. The absorbance was then read at 560 nm after 1 h incubation.

2.4.4. PopMsrA activity. The activity of MsrA in the presence of popTrx*h* was estimated by following the NADPH oxidation at 340 nm in the presence of either atNTRB system or the GSH/Grx system. In the first case, a 500 μl cuvette contained: 30 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 μM NADPH, 0.8 μM atNTRB, 16 μM popTrx*h*3, 10 mM L-MetSO and 3.5 μM popMsrA. To test popTrx*h*4, the 500 μl reaction mixture contained: 30 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 μM NADPH, 1 mM GSH, 0.5 IU glutathione reductase, 6 μM popGrx, 16 μM popTrx*h*4, 10 mM of L-MetSO and 3.5 μM popMsrA. In both cases, the reactions were initiated by adding popTrx*h*s.

2.5. HED reduction

The reduction of HED (7 mM) was tested by following the oxidation of NADPH as described in [11].

2.6. Thiol content titration

The thiol content of each protein preparation was measured using the DTNB procedure as described in [6].

3. Results and discussion

The search for Trx sequences in the poplar EST database led us to identify sequences corresponding to a putative full-length Trx called popTrx*h*4 and two putative full-length Trx-like proteins called popCXXS1 and popCXXS3 [10]. All known poplar Trxs *h* are members of the groups defined with *A. thaliana* sequences [10]. PopTrx*h*4, popCXXS1 and popCXXS3 are related to atTrx*h*9, atCXXS1 and atCXXS2 present in the third Trx *h* subgroup [1,5] (Fig. 1). PopTrx*h*4 exhibits the classical Trx active site WCGPC (Fig. 2A), whereas popCXXS1 and popCXXS3 exhibit an unusual active site WCMPS, analog of the *Arabidopsis* WC[I/L]PS active site (Fig. 2B). Since data concerning the biochemical properties of the third Trx *h* subgroup members are particularly scarce (except for a paper [5] which describes the poor reactivity of members of this group with *E. coli* NTR), we have overexpressed two of these sequences in *E. coli*.

Using DTNB as a substrate, we have observed that recombinant atNTRB is unable to reduce popTrx*h*4 even by increasing either the Trx or the NTR concentration (data not shown). Similar results were obtained using either *E. coli*

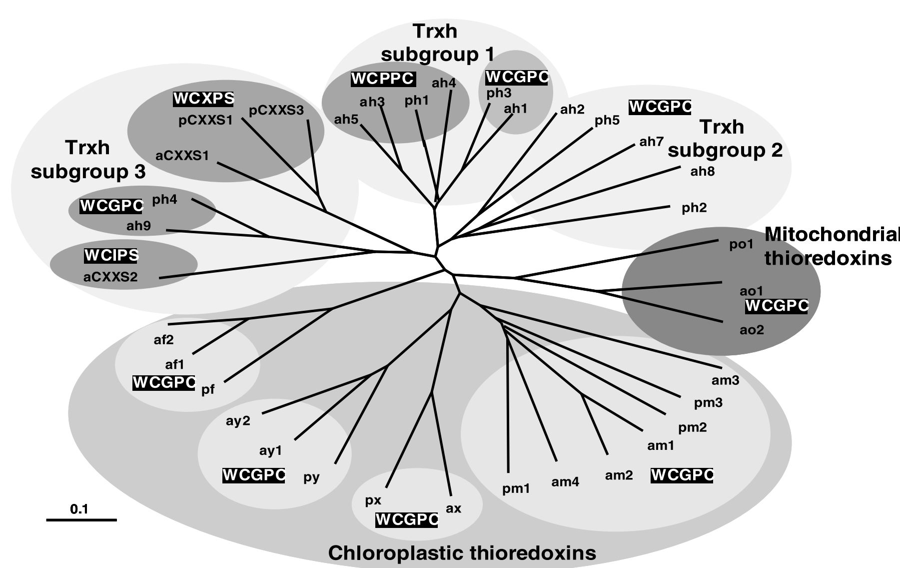

Fig. 1. Phylogenic tree of *Populus trichocarpa* cv. *Trichobel* and *A. thaliana* Trxs. Accession numbers and codes are as follows. *P. trichocarpa* cv. *Trichobel* (p): p*h*1 (AF483625); p*h*2 (AF483266); p*h*3 (BU822062); p*h*4 (BU835000); p*h*5 (BU869308); pCXXS1 (CA823821); pCXXS3 (BU874060); pm1 (BU831733); pm2 (BU879251); pm3 (BU867024); pf (BU827319); po (BU834909); px (866714); py (BU816567); *A. thaliana*: ah1 (P29448); ah2 (S58123); ah3 (S58118); ah4 (S58119); ah5 (S58120); ah7 (AAD39316); ah8 (AAG52561); ah9 (AAG51342), aCXXS1 (AF144390); aCXXS2 (ATU35639); ao1 (AAC12840); ao2 (AF396650); am1 (048737); am2 (AAF15949); am3 (AAF15950); am4 (Q9SEU6); af1 (Q9XFH8); af2 (Q9XFH9); ax (AAF15952); ay1 (NP_175021); ay2 (NP_177802).

A

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ph4             ---------MGLCLD-KYKRDADNDELHVEFAGGNVHLITTKESWDQKLSEASRDGKIVL 50
ah9             ---------MGSCVS-KGKGDDDS-VHNVEFSGGNVHLITTKESWDDKLAEADRDGKIVV 49
nh              MGITDMVHSLFSCFKTRSTNNDDDSSHNVEFAGGNVCLITTKESWDQKLAEANKEGKIVI 60
oh              ---------MGGCVG---KGRRHIEEDKLDFKGGNVHVITSKEDWDRKIEEANKDGKIVV 48
pch             ---------MGGCVG---KDR-GIVEDKLDFKGGNVHVITTKEDWDQKIAEANKDGKIVV 47
zh              ---------MGGCAG---KVR-RDDEEKLDFKGGNVHIITSNEGWDQKIAEANRDGKTVV 47
                            *     .        :::* **** :**::*.** *: **.::** *:

ph4             ANFSATWCGPCRQIAPFYNELSEKYPSLLFLLVDVDELSDLSTSWEIKATPTFFFLRDGK 110
ah9             ANFSATWCGPCKIVAPFFIELSEKHSSLMFLLVDVDELSDFSSSWDIKATPTFFFLKNGQ 109
nh              ANFSASWCGPCRMIAPFYCELSEKYLSLMFLTVDVDELTEFSSSWDIKATPTFFFLKDSQ 120
oh              ANFSASWCGPCRVIAPIYAEMSKTYPQLMFLTIDVDDLMDFSSSWDIRAKPTFFFIKNEK 108
ph              ANFSASWCGPCRVIAPVYAEMSKTYPQLMFLTIDVDDLVDFSSTWDIRATPTFFFLKNGQ 107
zh              ANFSASWCGPCRVIAPVYAEMSKTYPQLMFLTIDVDDLMDFSSSWDIRATPTFFFLKNGQ 107
                *****:*****: :**.: *:*:.: .*:** :***:* ::*::*:*:*.*****::: :

ph4             QLEKLVGANKPELQKKITAIVDSLPPSDK--- 139
ah9             QIGKLVGANKPELQKKVTSIIDSVPESPQRP- 140
nh              QIDKLVGANKPELQKKITAIADTQVVCETQPQ 152
oh              QVDKLVGANKPELEKKVQALADGS-------- 132
ph              QIDKLVGANKPELEKKVQALGDGS-------- 131
zh              QIDKLVGANKPELEKKVLAAADASTS------ 133
                *: **********:**: :  *
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B

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pCXXS3          --MESQEQQPKSRVIKVESVESWDFYITQATNQACPIVVHFTALWCMPSVAMNPVFEELA 58
pCXXS1          --MAGHSQVIKTRVVRIDSEKSWDFFINQATNKECPVVVHFTACWCMPSVAMNPFFEEVA 58
aCXXS1          ----------MARVVKIDSAESWNFYVSQAKNQNCPIVAHFTALWCIPSVFMNSFFEELA 50
hCXXS           --METQEQQAKSRVVKVDSVESWDFYVTQANNQGCPIVVHFTASWCIPSVAMNPFFEELA 58
zCXXS           MEIQHHRGLGNSKVVKVQSEEAWDLFTDQASNEGRPVVAHFGASWCVTSLSMNYKFEELA 60
                           ::*::::* ::*:::  **.*:  *:*.** * **:.*: **  ***:*

pCXXS3          SSYPDGLFLIVDVDAVKEVATKMEVKAMPTFLLMKDGAQVDKIVGANPEEIRKRIDGFVQ 118
pCXXS1          SNYKHILFLSVDVDEVKEIATRMEVKAMPTFLLMMGGARVDKLVGANPEEVRRRIGGFVH 118
aCXXS1          FNYKDALFLIVDVDEVKEVASQLEVKAMPTFLFLKDGNAMDKLVGANPDEIKKRVDGFVQ 110
hCXXS           SAYPDVLFLAVDVDEVKEVASKLEVKAMPTFVLMKDGAQIDRLVGANPEEIRKRIGGFAQ 118
zCXXS           QTHPEVLFLYVDVDDVQSVSSRYGVKAMPTFFLIKSKEVVGKIVGANPDEVKKLVDASAE 120
                  : . *** **** *:.::::  *******.:: .   :.::*****:*::: :.. ..

pCXXS3          SIRAYNA-- 125
pCXXS1          TIHGYKAI- 126
aCXXS1          SSRVVHIA- 118
hCXXS           SIRVAVA-- 125
zCXXS           PLETQIVVE 129
                . .
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Fig. 2. Alignment of popTrx*h*4 (A) and popCXXS3 (B) with several plant counterparts. Accession number and codes are as follows. ph4: *P. trichocarpa* (BU835000); ah9: *A. thaliana* (AAG51342); nh: *Nicotiana tabacum* (AF435818); oh: *Oryza sativa* (AF435817); pch: *Phalaris coerulescens* (AF159388); zh: *Zea mays* (AF435816); pCXXS1: *P. trichocarpa* (CA823821); pCXXS3: *P. trichocarpa* (BU874060); aCXXS1: *A. thaliana* (AF144390); hCXXS: *Hevea brasiliensis*; zCXXS: *Z. mays* (AY105925).

NTR or the mitochondrial atNTRA. PopPrxQ is a recently characterized poplar Prx localized in chloroplasts. This protein is reduced by Trxs but is not able to accept electrons from Grx (Rouhier et al., in preparation). We have used this property to demonstrate the reduction of popTrx*h*4 by popGrx (Fig. 3A). In similar experiments performed with popTrx*h*3 and popTrx*h*2, two members of different subgroups of Trx *h* (Fig. 1), no activity has been detected in the popGrx/popPrxQ system (Fig. 3A). Likewise, popGrx is not able to reduce the WCPPC popTrx*h*1. The interactions between popTrx*h*4 and the three described types of *E. coli* Grxs [14] have been investigated using the popPrxQ system (Fig. 3B). EcGrx1 and ecGrx3 are as active as popGrx in the PrxQ–popTrx*h*4 system. The activity measured in presence of ecGrx2 is about two thirds lower compared to the rates recorded with either ecGrx1 or ecGrx3. The fact that ecGrx2 is an atypical Grx, with a structure similar to GST [14], could also explain the different interaction between this protein and popTrx*h*4.

As PrxQ is a chloroplastic enzyme, the system is likely to be non-physiological since popTrx*h*4 is presumably a cytosolic enzyme. We have thus also analyzed the possible interactions of popTrx*h*4 with a cytosolic MsrA (popMsrA) recently characterized in poplar (Rouhier et al., in preparation). PopTrx*h*4 was also able to reduce this protein, the activity detected being

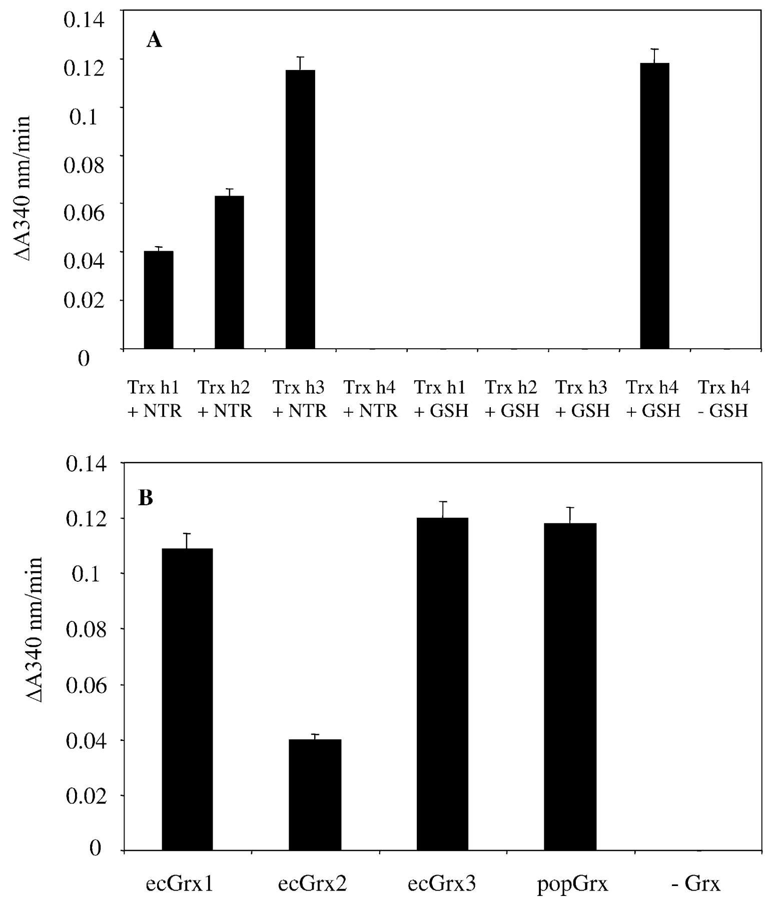

Fig. 3. Activity of popPrxQ in presence of different Trxs and Grxs. A: popPrxQ (2 μM) was incubated with several poplar Trxs (16 μM) in presence of either the NTR system (200 μM NADPH, 500 μM H_2O_2, 0.8 μM atNTRB) or the GSH/Grx sytem (200 μM NADPH, 1 mM GSH, 0.5 IU glutathione reductase, 6 μM popGrx, 500 μM H_2O_2). NADPH oxidation was measured at 340 nm as absorbance change per minute. B: popPrxQ (2 μM) was incubated with several *E. coli* and poplar Grxs (6 μM) in presence of 16 μM popTrx*h*4, 200 μM NADPH, 1 mM GSH, 0.5 IU glutathione reductase, and 500 μM H_2O_2. NADPH oxidation was measured at 340 nm as absorbance change per minute. Each point is the mean ± S.D. of three determinations.

similar to the one obtained in presence of the control popTrx*h*3 (Fig. 4). In the same way, popTrx*h*4 is also able to reduce PrxII, a cytosolic Prx able to accept both Trx and Grx as electrons donor [6] (data not shown).

In order to characterize the other members of Trx *h* third subgroup in poplar, popCXXS1 and popCXXS3 were also overexpressed in *E. coli*. Despite several attempts using various production and renaturation conditions, the insolubility of the preparation prevented further characterization of popCXXS1. On the other hand, the overexpression of popCXXS3 in *E. coli* led to a soluble preparation and the resulting protein could be purified to homogeneity. Recombinant popCXXS3 is not able to reduce insulin in the presence of DTT ([15], data not shown). Furthermore, no activity could be detected in the widely used 'DTNB/NTR' test in presence of popCXXS3 at concentrations as high as 50 μM (data not shown). The thiol content of popCXXS3 was determined using the DTNB method. Nearly two thiols (1.8) are titrated in the native recombinant protein showing the absence of disulfide formation between the two cysteines present in popCXXS3. We also tested the activity of popCXXS3 in the glutathione: HED transhydrogenase assay, a test largely used to assess Grx activity. In this assay, Grx catalyzes the reduction of a mixed disulfide bridge between glutathione and HED [16]. PopCXXS3 is active in this test suggesting that this protein exhibits a Grx-like activity (Fig. 5). PopTrx*h*3, used as control, was not active in presence of GSH in the same experimental conditions and was active in the HED test only in presence of atNTRB. The catalytic activities of both Trxs were similar in this test, but nearly 100-fold lower than those detected with popGrx. This difference is probably due to the low affinity of Trxs vs. HED [16].

To test for possible contamination of recombinant popCXXS3 preparation by *E. coli* Grxs, the activity of popCXXS3 was evaluated using either the popTrx*h*4/popPrxQ or popPrxII systems. In both systems, Grx would be required to promote activity (see above; [6]). In both cases, popCXXS3 is fully inactive even at concentrations as high as 160 μM, suggesting the absence of *E. coli* Grx in the popCXXS3 preparation.

The evidence that a specific subgroup of Trx *h* is not reduced via NTR but rather through the Grx/GSH system, increases the complexity of the cytosolic thiol reducing pathways (Fig. 6). Until now, it was usually admitted that in plant cytosol, both systems, i.e. NTR/Trx and GR/GSH/Grx, coexist without direct interconnection. Based on our results, it seems that both systems are probably interconnected with electron transfer from GSH (via Grx) through the members of the third Trx *h* subgroup. Since the three bicysteinic *E. coli* Grxs are able to reduce popTrx*h*4, it is likely that other

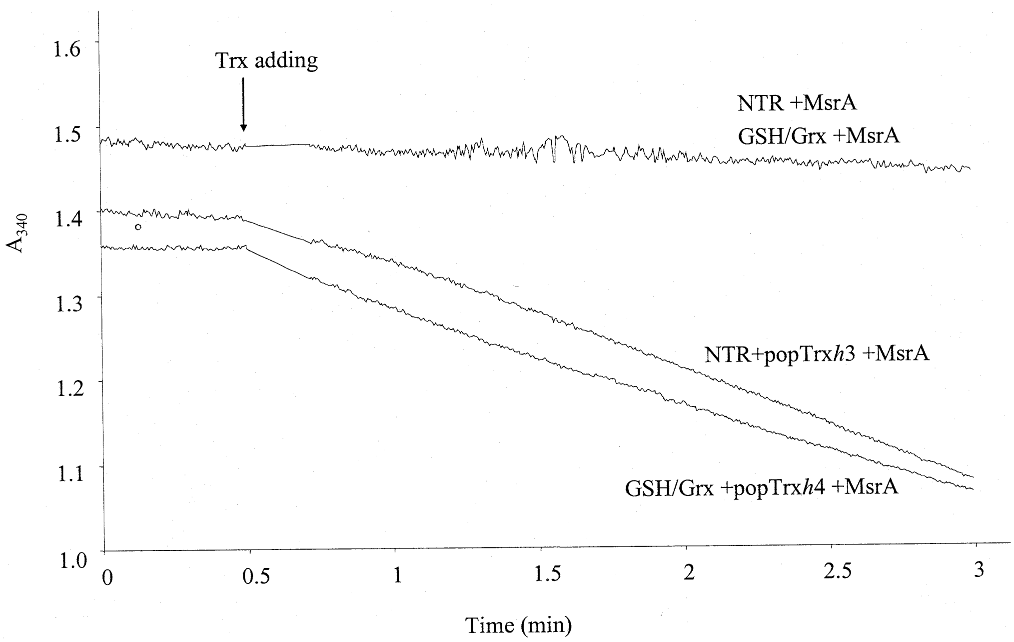

Fig. 4. Activity of popMsrA in presence of either popTrx*h*4 or popTrx*h*3. popMsrA (3.5 μM) was incubated in presence of either 30 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 μM NADPH, 0.8 μM atNTRB, 16 μM popTrx*h*3, 10 mM L-MetSO or 30 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 μM NADPH, 1 mM GSH, 0.5 IU glutathione reductase, 6 μM popGrx, 16 μM popTrx*h*4, 10 mM of L-MetSO. NADPH oxidation was measured at 340 nm.

poplar Grx isoforms should also be able to interact with popTrx*h*4. As most characterized Trxs have a redox potential of ca −290 mV and Grxs are more electropositive (ca −200 mV), the reduction of a Trx-like molecule by Grx is supposed to be an unfavorable reaction unless the redox potential of Trx*h*4 is quite different from those usually described or the catalytic mechanism differs notably from the traditional dithiol/disulfide exchange. We are currently investigating these parameters in order to answer these questions.

In contrast to popTrx*h*4, popCXXS3 is active in the GSH/HED test and represents the first described example in plants of this kind of protein, i.e. a Trx-like protein with a Grx-like activity. The obtained results suggest that popCXXS3 could exhibit deglutathionylation activity using a monothiol mechanism as suggested previously for some Grx isoforms [14]. In this mechanism, the thiolate of Grx initiates a nucleophilic attack on the mixed disulfide of a protein thiol with GSH. A new disulfide between Grx and GSH is formed, which could then be reduced by GSH leading to the formation of GSSG and to the release of the reduced Grx. Nevertheless, the large difference of catalytic efficiency between popCXXS3 and Grx in the HED test suggests that the physiological function of popCXXS3 could not be deglutathionylation.

The information about potential relationships between Trx and GSH/Grx systems are scarce particularly in plants. It was recently reported that the human Trx activity could be regu-

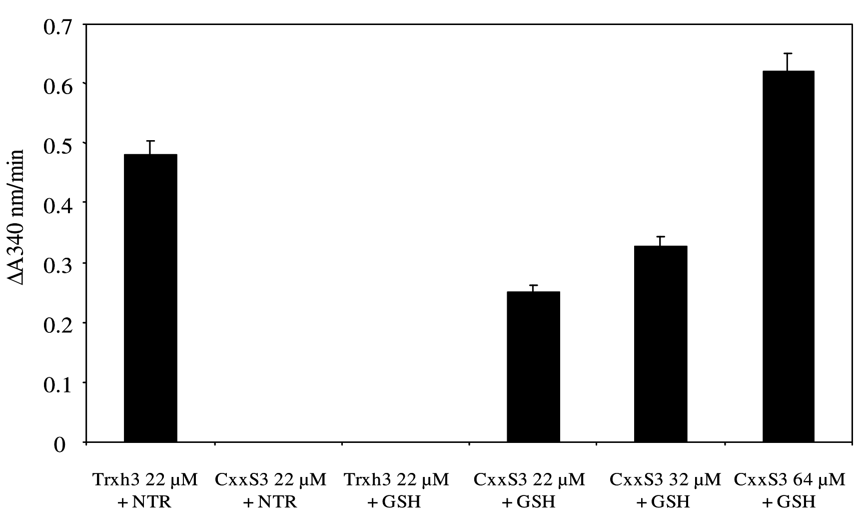

Fig. 5. Grx-like activity of popCXXS3. Experiments have been performed using various concentrations of popCXXS3 (22, 32 and 64 μM) with either 200 μM NADPH, 1 mM GSH, 0.5 IU glutathione reductase, and 7mM HED or 200 μM NADPH, 0.8 μM atNTRB and 7 mM HED. NADPH oxidation was measured at 340 nm as absorbance change per minute. popTrxh3 (22 μM) was also used as control. Each point is the mean ± S.D. of three determinations.

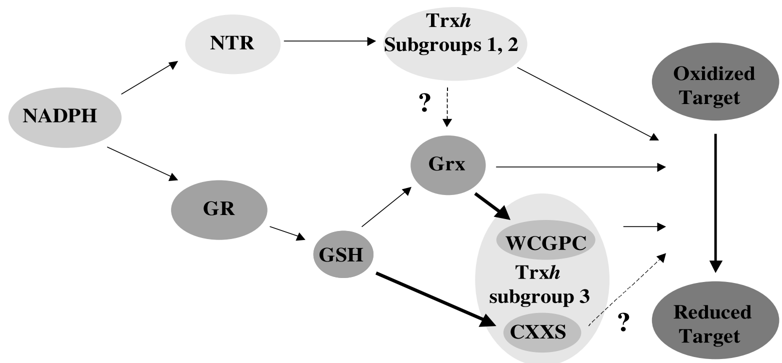

Fig. 6. Alternative cytosolic reducing pathways. The reducing power is provided by NADPH and transmitted by dithiol–disulfide exchange via the NTR/Trx and the GSH/Grx systems to reduce target proteins. Dotted lines represent hypothetical interactions between the Trx and Grx systems and their target proteins.

lated by glutathionylation [17]. In *Saccharomyces cerevisiae*, the Trx system remains reduced independently of the GSH/Grx system [18]. Nevertheless, an electron transfer from Trx to Grx could not be excluded. Indeed, it was recently reported that GSH is probably not the physiological reducing agent of yeast Grx5 whereas *E. coli* Trx reduces efficiently this protein [19]. The physiological significances of these interconnections remain to be investigated.

Acknowledgements: We thank Dr. Vlamis Gardikas for the gift of ecGrxs. This work was supported by grants of BQR and Région Lorraine.

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