Characterization of thioredoxin *y*, a new type of thioredoxin identified in the genome of *Chlamydomonas reinhardtii*

S.D. Lemaire[a,*], V. Collin[a], E. Keryer[a], A. Quesada[b], M. Miginiac-Maslow[a]

[a] *Institut de Biotechnologie des Plantes, UMR 8618 CNRS, Université Paris-Sud, Bâtiment 630, 91405 Orsay Cedex, France*
[b] *Departamento de Bioquímica y Biología Molecular, Faculdad de Veterinaria, Avenida de la Universidad s/n, 10071 Cáceres, Spain*

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Abstract The sequencing of the *Arabidopsis* genome revealed a multiplicity of thioredoxins (TRX), ubiquitous protein disulfide oxido-reductases. We have analyzed the TRX family in the genome of the unicellular green alga *Chlamydomonas reinhardtii* and identified eight different thioredoxins for which we have cloned and sequenced the corresponding cDNAs. One of these TRXs represents a new type that we named TRX *y*. This most probably chloroplastic TRX is highly conserved in photosynthetic organisms. The biochemical characterization of the recombinant protein shows that it exhibits a thermal stability profile and specificity toward target enzymes completely different from those of TRXs characterized so far.

Key words: Fructose-1,6-bisphosphate phosphatase; NADP-malate dehydrogenase; Peroxiredoxin; Thioredoxin; *Chlamydomonas reinhardtii*

1. Introduction

Thioredoxins (TRX) are ubiquitous protein disulfide oxido-reductases involved in a wide range of biochemical pathways [1,2]. In plants, two chloroplastic isoforms called TRX*f* and TRX*m* were known so far. They are reduced in the light by a ferredoxin-dependent thioredoxin reductase (FTR) and participate in the reductive activation of several key enzymes of carbon metabolism such as fructose-1,6-bisphosphatase (FBPase) or NADP-malate dehydrogenase (NADP-MDH). More recently, several chloroplastic TRX-dependent peroxiredoxins have been identified [3] and we have isolated a chloroplastic 2-cys peroxiredoxin (2-cys PRX) from *Chlamydomonas* [4]. Cytosolic TRX isoforms, called TRX*h*, whose reduction proceeds via an NADPH-dependent thioredoxin reductase (NTR), are involved in the mobilization of seed reserves during germination [5,6] and in self-incompatibility [7] but their function in green leaves and algae remains unknown. The completion of the genome of *Arabidopsis thaliana* made it possible to identify new TRX types, one located in mitochondria (TRX*o* [8]) and another presumably in chloroplasts (TRX*x* [9]). The analysis of the *Arabidopsis* genome also revealed the presence of several TRX isoforms for each type: eight TRX*h*, two TRX*f*, four TRX*m*, two TRX*o* and one TRX*x* [10], raising questions about their specificity or redundancy. Apart from *A. thaliana*, *Chlamydomonas reinhardtii* is one of the major photosynthetic eukaryotes used as a model organism. It is a unicellular green alga which presents many advantages making it a very good model in particular for the study of photosynthesis [11]. In our previous work, we purified a TRX*m* (Ch2) and a TRX*h* (Ch1) from *Chlamydomonas*, cloned the corresponding cDNAs and genes, studied their expression, analyzed the biochemical properties of the wild-type proteins and a set of mutant TRXs and determined their three-dimensional structures (reviewed in [12]). However, nothing was known about the total number of *Chlamydomonas* TRXs. Recently, the *Chlamydomonas* genome project reached an advanced stage, with a very large expressed sequence tag (EST) database being available. Moreover, the first draft of the complete *Chlamydomonas* genome has been available since February 4, 2003. Taking advantage of these facilities, we were able to identify eight different TRXs in *Chlamydomonas* and clone and sequence the corresponding cDNAs. One of these TRXs belongs to a new type, which we designated TRX*y*. We show that this new TRX, presumably chloroplastic, is highly conserved in photosynthetic organisms. We have expressed and purified recombinant TRX*y* and found that it exhibits a specific thermal stability profile. Moreover, it was able to activate FBPase but not NADP-MDH, a feature completely different from that of all known TRXs.

2. Material and methods

2.1. cDNA cloning and sequencing

All cDNA clones were obtained from the Kazusa DNA Research Institute, sequenced and sequences deposited in the GenBank database. *Chlamydomonas* TRX*h1*, initially called Ch1, was renamed *Cr*TRX*h1*. TRX*m*, initially called Ch2, was renamed *Cr*TRX*m*.

2.2. Purification of recombinant TRXy

The coding region of mature *Cr*TRX*y* was amplified by polymerase chain reaction (PCR) in order to substitute Thr38 by Met and cloned in pSBET vector between *Nde*I and *Bam*HI restriction sites [13]. After transformation of this vector into *Escherichia coli* BL21(DE3) strain, a kanamycin-resistant clone was propagated to 5 l in LB medium for 16 h at 30°C. Cells were harvested by centrifugation (10 min, 5000×*g*), resuspended in a minimum volume of ice-cold extraction buffer (30 mM Tris–HCl, pH 7.9; 1 mM EDTA) supplemented with Complete™ protease inhibitor cocktail (Roche). All further steps were performed essentially as described [14], but with a milder heat treatment of the crude extract (60°C, 1 min 30 s). The protein was recov-

*Corresponding author. Fax: (33)-1-69 15 34 23.
E-mail address: lemaire@ibp.u-psud.fr (S.D. Lemaire).

Abbreviations: EST, expressed sequence tag; FBPase, fructose-1,6-bisphosphate phosphatase; NADP-MDH, NADP-malate dehydrogenase; 2-cys PRX, 2-cys peroxiredoxin; TRX, thioredoxin; NTR, NADPH thioredoxin reductase

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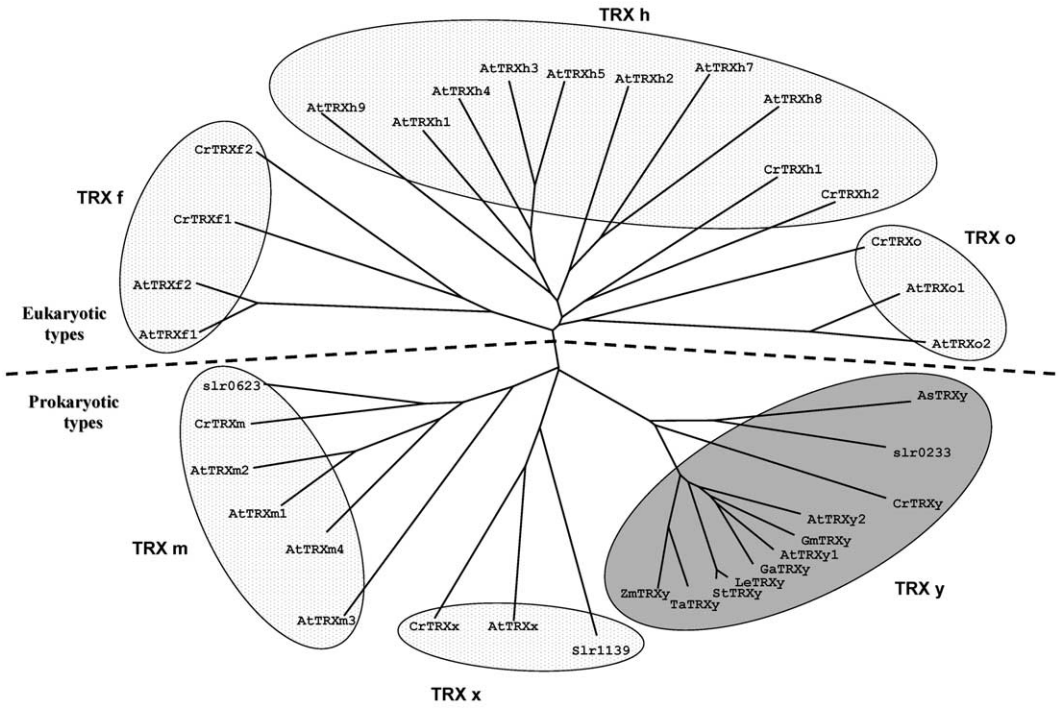

Fig. 1. The TRX family in *Chlamydomonas*. Phylogenetic tree of the TRX families in *C. reinhardtii*, *A. thaliana*, *Synechocystis* PCC6803 and TRX*y* from different organisms. The unrooted tree was constructed with Clustal X. Gaps were excluded. Sequences were deduced using EST clustering when genome sequences were not available, only one EST accession number from the cluster is given. Accession numbers: Cr: *C. reinhardtii h1*, P80028; *h2*, AY184797; *o*, AY184798; *m*, P23400; *f1*, AY184800; *f2*, AV622215; *x*, AY184799; *y*, AY184796; At: *A. thaliana h1*, Z14084; *h2*, Z35475; *h3*, Z35474; *h4*, Z35473; *h5*, Z35476; *h7*, AAD39316; *h8*, AAG52561; *h9*, AAG51342; *o1*, AAC12840; *o2*, AF396650; *m1*, O48737; *m2*, Q9SEU8; *m3*, Q9SEU7; *m4*, Q9SEU6; *f1*, Q9XFH8; *f2*, Q9XFH9; *x*, AAF15952; *y1*, AAF04439 corrected for intron splicing; *y2*, AAM91085 corrected with EST AY128276; *Synechocystis* PCC6803 slr0623, slr1139 and slr0233 (sll1057 was omitted since it is not conserved in other organisms). As, *Anabaena* sp., BAB73592; Ga, *Gossypium arboretum*, BF272249; Gm, *Glycine max*, BE800303; Le, *Lycopersicon esculentum*, BG643685; Ta, *Triticum aestivum*, BF485490; St, *Solanum tuberosum*, BM111202; Zm, *Zea mays*, BI431154.

ered from the anion exchange column in the non-adsorbed fraction. The final preparation was stored at 4°C.

2.3. Purification of other enzymes and biochemical assays

Purification and enzymatic assays were performed as described for recombinant pea FBPase [15], sorghum NADP-MDH [16], *Chlamydomonas* 2-cys PRX [4], *Arabidopsis* NTR [17], and other TRXs [14]. The coding region of mature *Chlamydomonas* NADP-MDH was PCR amplified in order to substitute Ala32 by Met and cloned into Pet11d vector (Novagen) between *Nco*I and *Bam*HI restrictions sites [18]. The purification of the recombinant protein followed the procedures described for the sorghum protein except that it was eluted from Matrex RedA column with a 0 to 3 M NaCl gradient.

2.4. pH- and temperature-dependent aggregation assays

Aggregation assays were performed as described [19].

3. Results and discussion

When this analysis was initiated the genome sequence of *C. reinhardtii* was not yet available but a very large EST database had already been constructed. This database has the advantage that it includes cDNA libraries from cultures made under different nutritional and stress conditions [20]. An analysis based on sequence homology searches and EST clustering allowed us to identify eight different thioredoxins. No additional TRX gene was found in the *Chlamydomonas* genome database, when it became available. *Chlamydomonas* thioredoxin family contains two TRX*f*, one TRX*m*, two TRX*h*, one TRX*o*, one TRX*x*, but also an additional TRX that does not belong to the types described so far. We have called it TRX*y* to continue the nomenclature started with TRX*x*. A phylogenetic tree containing the TRX families of *Chlamydomonas*, *Arabidopsis* and *Synechocystis* is presented in Fig. 1. The presence of a new type of TRX was surprising considering that the *Arabidopsis* genome had already been analyzed. However, a database search allowed us to identify two genes encoding TRX*y* homologues in *Arabidopsis* that we called *At*TRX*y1* and *At*TRX*y2*. These genes had initially been annotated as TRX-like proteins and were not considered typical TRXs. Further homology searches and EST clustering made it possible to identify TRX*y* in many photosynthetic organisms. An alignment of these sequences is presented in Fig. 2. One of the most striking features is the very high conservation of TRX*y*. Such conservation is quite unusual for TRXs since generally only the active site and a few additional amino acids are conserved. This suggests that TRX*y* would have a very specific function in photosynthetic organisms. All eukaryotic sequences possess an N-terminal extension that is not found in cyanobacteria. This extension is likely to correspond to a putative transit peptide for targeting to the chloroplast. Cell sorting prediction programs are not adapted to *Chlamydomonas* but are very well suited for *Arabidopsis* sequences. Both *At*TRX*y* sequences are predicted to be addressed to the chloroplast with a very high score by Predotar 0.5, iPSORT and TargetP 1.01 (links available at http://www.expasy.ch). All these observations suggest that TRX*y* constitutes a new type of chloroplastic TRXs.

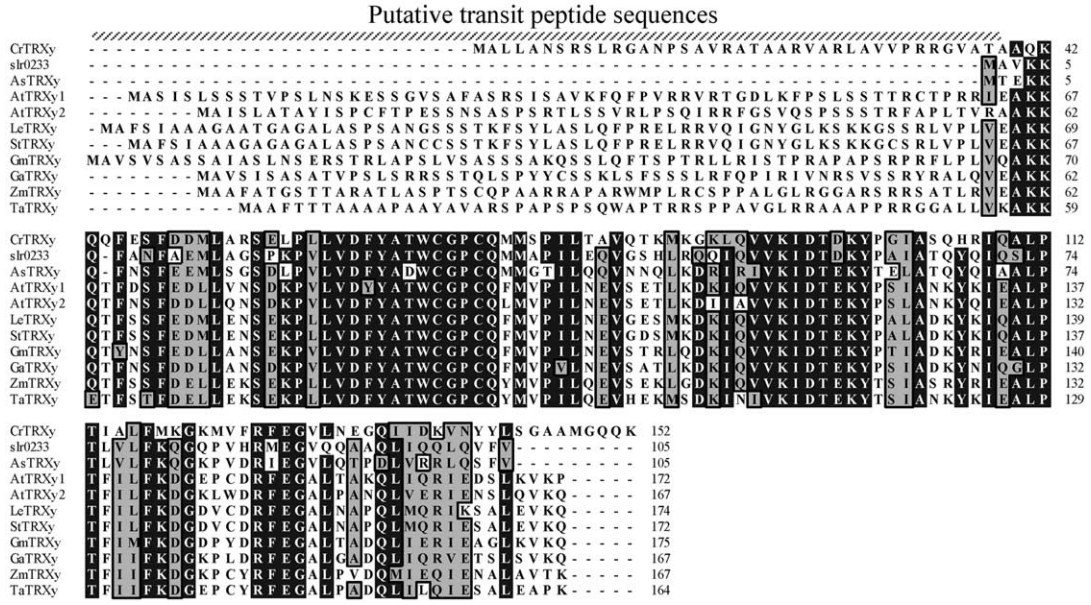

Fig. 2. Multiple sequence alignment of TRX*y* from different organisms. The proteins were aligned with Clustal W and corrected manually for N-terminal extensions. Residues boxed in black correspond to 80% identity. Residues boxed in gray correspond to 80% conservation with the PAM120 matrix. Sequences were deduced using EST clustering when genome sequences were not available. Accession numbers and abbreviations as in Fig. 1.

Chlamydomonas contains a limited number of TRXs compared to *Arabidopsis*: all TRX types are represented, but with fewer isoforms for each type. This might be linked to the fact that many TRXs found in higher plants are involved in functions that are not necessary in algae, such as seed development [4,5] or auto-incompatibility [7]. Thus, *Chlamydomonas* appears as a model of choice for the study of basic functions of TRXs. Moreover, the small number of isoforms in each type renders a reverse genetics approach more feasible.

TRXs are usually described as being heat-stable proteins. TRX purification procedures take advantage of this feature and include a heat shock at 75–80°C. In the case of *Cr*TRX*y*, the protein turned to be more heat-sensitive and we had to lower the heat shock temperature to 60°C. *Cr*TRX*y* also appeared particularly sensitive to proteolysis, thus the extraction buffer had to be supplemented with a cocktail of protease inhibitors. Since protease sensitivity and thermal stability are often correlated [21], this prompted us to analyze the pH- and temperature-dependent aggregation of purified *Cr*TRX*y* (Fig. 3A). Aggregation of *E. coli* TRX, *Cr*TRX*m* and *Cr*TRX*h1* occurs in the pH ranges of 3.5–5.5, 4–6 and 4.5–7.5, respectively [19]. The temperature where half of the protein is aggregated ($T_{agg1/2}$), plotted as a function of pH (Fig. 3B), shows that this is not the case for *Cr*TRX*y*, which precipitates above 4.5, with aggregation being more pronounced above pH 7, a pH range where other TRXs are stable. We have also observed that purified *Cr*TRX*y* loses activity after long-term storage at −20°C in 30 mM Tris–HCl, pH 7.9 but remains active when stored at 4°C in the same buffer (data not shown). This phenomenon might be similar to the cold denaturation observed at certain pH values for *Cr*TRX*h1* [22].

We have also analyzed the reactivity of *Cr*TRX*y* with three chloroplastic target enzymes, namely FBPase, NADP-MDH and 2-cys PRX, and its ability to be reduced by NTR.

Chloroplastic FBPase is known to be exclusively reduced by

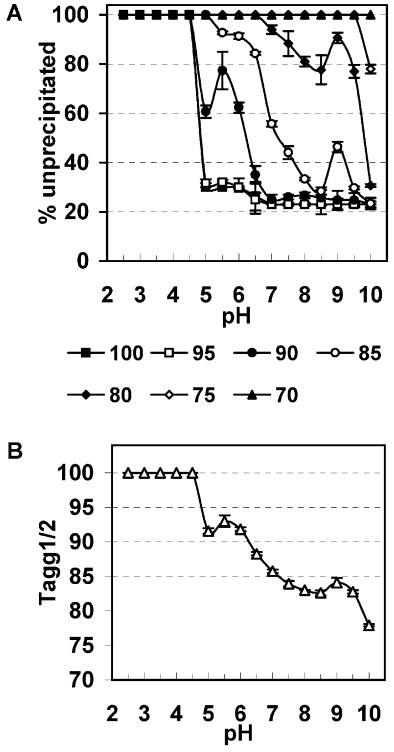

Fig. 3. Aggregation properties of *Chlamydomonas* TRX*y*. A: pH and temperature dependence of *Cr*TRX*y* aggregation. B: pH dependence of $T_{agg1/2}$ for *Cr*TRX*y*. The aggregation was tested by heating 100 μM TRX solutions at the desired temperature for 3 min and cooling to 5°C for 3 min. After centrifugation at 12 000×*g* for 4 min to remove insoluble aggregates, the absorbance of the supernatant at 280 nm was measured. $T_{agg1/2}$ represents the temperature where half of the protein is aggregated. The mean ± S.D. derived from three independent experiments are plotted.

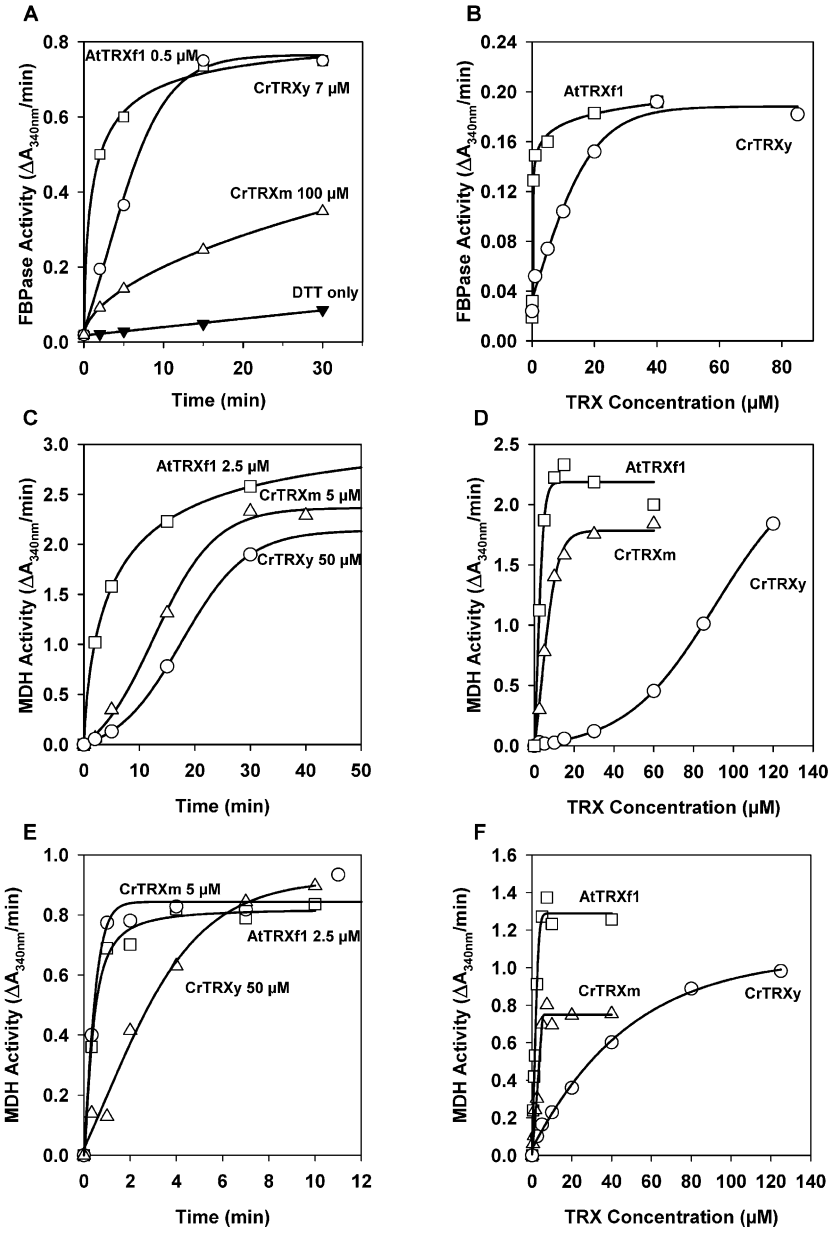

Fig. 4. Sorghum NADP-MDH, *Chlamydomonas* NADP-MDH and FBPase activation by *Cr*TRX*y*, *Cr*TRX*m* and *At*TRX*f1*. A: Time course of activation of pea FBPase in the presence of 0.4 µM *At*TRX*f1*, 7 µM *Cr*TRX*y*, or 100 µM *Cr*TRX*m* (concentrations corresponding to the $S_{0.5}$). B: Activation rate of FBPase as a function of thioredoxin concentration. C: Time course of activation of sorghum NADP-MDH in the presence of 2.5 µM *At*TRX*f1*, 5 µM *Cr*TRX*m*, or 50 µM *Cr*TRX*y* (concentrations corresponding to the $S_{0.5}$, except for *Cr*TRX*y* for which this parameter could not be measured). D: Activation rate of sorghum NADP-MDH as a function of thioredoxin concentration. E: Time course of activation of *Chlamydomonas* NADP-MDH in the presence of 2.5 µM *At*TRX*f1*, 5 µM *Cr*TRX*m*, or 50 µM *Cr*TRX*y*. F: Activation rate of *Chlamydomonas* NADP-MDH as a function of thioredoxin concentration. All experiments were performed in the presence of 5 mM dithiothreitol.

TRX*f* [23]. Surprisingly, *Cr*TRX*y* was found to be active in this test (Fig. 4A,B). However, it was much less efficient than TRX*f* (Table 1). The affinity of *Cr*TRX*y* for FBPase, estimated by the $S_{0.5}$ value, was 10 times lower than that of *At*TRX*f1*. These measurements were performed with purified pea FBPase that is closely related to *Chlamydomonas* FBPase (data not shown). In particular, the position and environment of the regulatory cysteines are conserved in both enzymes [15,24]. Thus, the differences observed are likely to reflect reactivities of different TRX types rather than differences between *Arabidopsis* and *Chlamydomonas* FBPases. *Cr*TRX*y* is probably less abundant than TRX*f* since it has never been identified with standard biochemical approaches that allowed isolation of TRX*f*, TRX*m* and TRX*h*. The EST abundance, which can be used as a rough indication of the relative abundance of the proteins, also indicates that TRX*y* is less abundant. In *Chlamydomonas*, in standard culture conditions, seven ESTs are found for *Cr*TRX*y* compared to 36 for *Cr*TRX*f1*. Consequently, TRX*y* is probably not the in vivo activator of FBPase and is likely to have another function. However, it cannot be excluded that it could activate FBPase in specific conditions where TRX*f* is less abundant.

Table 1
Kinetic parameters for NADP-MDH and FBPase activation by different TRXs and reduction efficiency by NTR

	Sorghum NADP-MDH		*Chlamydomonas* NADP-MDH		FBPase		NTR
	$S_{0.5}$ (μM)	$t_{0.5}$ (min)	$S_{0.5}$ (μM)	$t_{0.5}$ (s)	$S_{0.5}$ (μM)	$t_{0.5}$ (min)	μmol NADPH oxidized/min
*Cr*TRX*y*	ND	ND	ND	ND	7	5	3.7
*At*TRX*f1*	2.5	3.7	1.8	25	0.4	0.9	ND
*Cr*TRX*m*	5	13.5	2.9	25	ND	ND	5.5
*Cr*TRX*h1*	NM	NM	NM	NM	NM	NM	38.1

ND: not determined, due to very low activity. NM: not measured. *Cr*TRX*h1* is used as a reference for NTR efficiency.

NADP-MDH does not show a high selectivity for TRXs in vitro and is considered a standard test for all types of TRXs [25]. The kinetic parameters of the activation of recombinant sorghum leaf NADP-MDH by different types of TRXs (Fig. 4C,D) show that TRX*f* is the most efficient activator of NADP-MDH, followed by TRX*m* (Table 1), in agreement with previously published data [14,26]. However, TRX*y* was unable to activate the enzyme, unless used at very high concentrations, whereas even the cytosolic *Cr*TRX*h* is reasonably efficient in this test [14]. Recently a cDNA encoding *Chlamydomonas* NADP-MDH was isolated [18]. Contrary to FBPase, *Chlamydomonas* NADP-MDH is different from its higher plant counterpart since it is missing the N-terminal regulatory disulfide while the other regulatory cysteines are present. Thus, even if *Cr*TRX*y* is unable to activate a higher plant NADP-MDH, it might have been able to activate the algal enzyme due to different regulation properties. We thus purified recombinant *Chlamydomonas* NADP-MDH and tested its reactivity with different TRXs (Fig. 4E,F). The algal enzyme was activated much more rapidly than the sorghum enzyme. This is consistent with the absence of an N-terminal disulfide bridge whose reduction is known to induce a rate-limiting slow conformational change in the higher plant enzyme [16]. On the other hand, the affinity of the different types of TRX for *Chlamydomonas* NADP-MDH is comparable to that observed for the sorghum enzyme (Table 1). Indeed, this enzyme could be activated by TRX*f* and TRX*m*, while the activation by *Cr*TRX*y* appeared quite inefficient as in the case of sorghum NADP-MDH.

Chlamydomonas chloroplastic 2-cys PRX (*Cr*PRX1) reduces alkyl hydroperoxides and hydrogen peroxide using reduced TRX as an electron donor. We tested the reduction of *t*-butyl hydroperoxide by *Cr*PRX1 in the presence of different types of TRXs (Fig. 5). All TRXs, including *Cr*TRX*y*, showed a similar efficiency of reduction of *Cr*PRX1. This is consistent with the lack of specificity we already observed in vitro for this PRX [4].

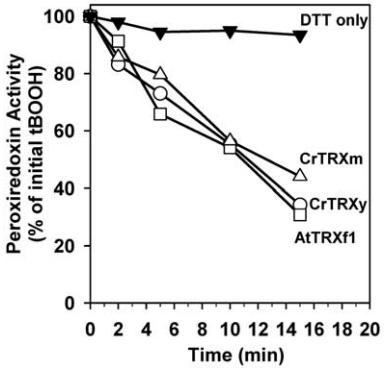

Fig. 5. Peroxidase activity of *Cr*PRX1 in the presence of *Cr*TRX*y*, *Cr*TRX*m* and *At*TRX*f1*. The concentration of *t*-butyl hydroperoxide (tBOOH) was measured colorimetrically and expressed as a percentage of initial concentration. Thioredoxins (16 μM) were reduced with 0.4 mM dithiothreitol.

NTR uses NADPH to reduce cytosolic and mitochondrial TRXs. In vitro cytosolic NTR reduces TRX*h* very efficiently but shows poor reduction efficiency with chloroplast TRXs that are reduced in vivo by FTR [14]. The comparison of the reduction rates of *Cr*TRX*y*, *Cr*TRX*m* and *Cr*TRX*h1* by NTR (Table 1) shows that *Cr*TRX*y* reduction is even less efficient than the reduction of *Cr*TRX*m*. This observation indicates that NTR is probably not the physiological reductant of *Cr*TRX*y* and is in accordance with the chloroplastic localization of *Cr*TRX*y* suggested by sequence analysis. However, this localization will have to be confirmed experimentally.

4. Conclusion

Chlamydomonas TRX*y* constitutes a new type of TRX, most probably chloroplastic, with unusual specificities for different known target enzymes. However, its real target probably remains to be identified.

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