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Evidence of non-functional redundancy between two pea *h*-type thioredoxins by specificity and stability studies

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ABSTRACT

The largest group of plant thioredoxins (TRXs) consists of the so-called *h*-type; their great number raises questions about their specific or redundant roles in plant cells. *Pisum sativum* thioredoxin *h*1 (PsTRX*h*1) and *Pisum sativum* thioredoxin *h*2 (PsTRX*h*2) are both *h*-type TRXs from pea (*Pisum sativum*) previously identified and biochemically characterized. While both are involved in redox regulation and show a high-sequence identity (60%), they display different behavior during *in vitro* and *in vivo* assays. In this work, we show that these two proteins display different specificity in the capturing of protein targets *in vitro*, by the use of a new stringent method. PsTRX*h*2 interacted with classical antioxidant proteins, whereas PsTRX*h*1 showed a completely different pattern of targeted proteins, and was able to capture a transcription factor. We also showed that the two proteins display very different thermal and chemical stabilities. We suggest that the differences in thermal and chemical stability point to a distinct and characteristic pattern of protein specificity.

Introduction

Thioredoxins (TRXs) belong to a family of proteins that are present in all living organisms, from bacteria to mammals and plants. A TRX is a disulfide oxido-reductase enzyme, typically a small (10–14 kDa), monomeric and heat-stable protein. Its catalytic mechanism involves a conserved peptide sequence, –WCG/PPC–, and it occurs *via* a reversible disulfide–dithiol reaction of the two –SH groups (Holmgren, 1989). Despite the wide range of variation in primary structures among all living organisms, the 3D-arrangement of TRXs is highly conserved (of a central β-sheet flanked by α-helices; Eklund et al., 1991). TRXs interact with a large number of targets, suggesting their involvement in redox control of many cellular processes (Buchanan and Balmer, 2005). However, different types of interactions are involved in TRX-target recognition, such as electrostatic or hydrophobic bonds (Wangensteen et al., 2001).

A wide range of genes encoding TRXs has been identified in plants, animals, fungi and bacteria (two and three genes in *Escherichia coli* and *Homo sapiens*, respectively). For instance, in *Arabidopsis*, about 40 genes encoding TRXs or TRX-related proteins have been described (Meyer et al., 2006). These genes have been classified on the basis of: (i) the intron position; (ii) the primary structures of the encoded products; and, (iii) the subcellular localization (Meyer et al., 2002). Thus, the TRXs *f*, *m*, *x* and *y* are plastidial (Buchanan 1980; Issakidis-Bourguet et al., 2001; Collin et al., 2003; 2004; Balmer et al., 2006), whereas TRXs *h* and *o* are localized in the cytoplasm or mitochondria (Florencio et al., 1988; Laloi et al., 2001; Gelhaye et al., 2004). Chloroplastidial TRXs are reduced by ferredoxin from photosynthetic electron transport *via* ferredoxin–thioredoxin reductase (FTR), but reduction of TRXs *o* and *h* depends on NADPH and involves NADPH-dependent thioredoxin reductases (NTR).

The most complex group of plant TRXs is the so-called *h*-type (Meyer et al., 2005). The specific functions of this group of TRXs are not well known due to the large number of isoforms that coexist in plant cells (about 10 isoforms in *Arabidopsis*; Meyer et al., 2006). Further, *in vitro* experiments (that is, enzymatic activities or target capturing) using *h*-type TRXs usually lead to similar results, even when TRXs from different groups are tested.

Abbreviations: CD, circular dichroism; GdmCl, guanidinium hydrochloride; PsTRX*h*1, *Pisum sativum* thioredoxin *h*1; PsTRX*h*2, *Pisum sativum* thioredoxin *h*2; T_m, thermal midpoint; TRX, thioredoxin; UV, ultraviolet

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On the other hand, experiments *in vivo* (that is, yeast complementation studies) have shown functional differentiation among *h*-type TRXs in *Arabidopsis* and *Pisum sativum* (Mouhaeb et al., 1998; Traverso et al., 2007a). However, no clear thermodynamic or proteomic data are available to address questions of functional specificity of the *h*-type TRXs.

To date, eight TRXs from *P. sativum* have been described. The pea *m*- and *f*-type groups were initially characterized as redox modulators of Calvin cycle enzymes in the chloroplast (López-Jaramillo et al., 1997; Pagano et al., 2000). However, the number of their potential roles has increased since they have been found in other locations rather than their classical chloroplastidial sites (Traverso et al., 2008). In addition, a new *o*-type mitochondrial pea TRX has been characterized (Barranco-Medina et al., 2007). The *h*-type cluster in pea is formed by at least four members: *Pisum sativum* thioredoxin *h*3 (PsTRX*h*3) and *Pisum sativum* thioredoxin *h*4 (PsTRX*h*4) are involved in seed germination events (Montrichard et al., 2003), and *Pisum sativum* thioredoxin *h*1 (PsTRX*h*1) and *Pisum sativum* thioredoxin *h*2 (PsTRX*h*2) seem to be involved in redox regulation throughout the whole plant (Traverso et al., 2007a).

PsTRX*h*1 and PsTRX*h*2 show reductive activity in nonspecific assays to reduce insulin molecules (Traverso et al., 2007a). However, while PsTRX*h*1 is able to complement the *S. cerevisiae* mutant (trx1Δ, trx2Δ) against hydrogen peroxide, PsTRX*h*2 causes a hypersensitive phenotype under the same conditions (Traverso et al., 2007a). Nevertheless, in the presence of ter-butyl hydroperoxide, another oxidant agent, PsTRX*h*2, produces a markedly better complementation in the mutant. Both proteins produce an antagonistic effect when interacting with pea chloroplast fructose-1,6-bisphosphatase (Traverso et al., 2007b). It is clear that more information about the two pea proteins is needed to understand their specificities and their interactions with other proteins.

To elucidate the reasons that the two proteins, PsTRX*h*1 and PsTRX*h*2, show different *in vitro* and *in vivo* behaviors, we used a two-approach study. First, we investigated the abilities of PsTRX*h*1 and PsTRX*h*2 to interact with pea proteins by using a new, highly stringent affinity chromatography method. Second, we explored the thermal and chemical stabilities of PsTRX*h*1 and PsTRX*h*2. Thus, although the similarity or identity between the two proteins is very high (76% and 60%, respectively), we show that PsTRX*h*1 is more stable than PsTRX*h*2 against heat or chemical denaturations. In addition, the different protein recognition pattern is consistent with our previous characterizations (Traverso et al., 2007a). Among all protein targets, PsTRX*h*2 interacted with classical antioxidant proteins, whereas PsTRX*h*1 was able to capture a pea transcription factor. We suggest that the different protein specificities are mirrored by the different biophysical properties.

Materials and methods

Protein purification, plant material, and growth conditions: The cDNAs from PsTRX*h*1 and PsTRX*h*2 (Traverso et al., 2007a) were cloned into pETm11 (Novagen) into the BamHI and HindIII sites. Proteins were over-expressed in *E. coli* BL21 as His-tagged proteins, and induced with IPTG at an absorbance at 600 nm of 0.6. Proteins were purified by Ni^{2+}-affinity chromatography in pre-packed HiTrap affinity columns (GE Healthcare) according to the manufacturer's instructions. Briefly, the purification protocol consisted of a lysis step (buffer containing 50 mM Tris at pH 8.0, 1% Triton X-100, 0.5 M NaCl, 1 mM β-mercaptoethanol, and 10 mM imidazole) to disrupt cells, followed by sonication and centrifugation to separate the cell pellet from the soluble fraction. The supernatant was loaded into the column, equilibrated in the same buffer, and eluted in a gradient with 1 M imidazole. The protein concentration was determined from extinction coefficient models (Gill and Hippel, 1989) and from the Bradford reaction (BioRad).

Pea (*Pisum sativum* L. cv Lincoln) seeds were germinated on vermiculite in plastic trays and grown in a growth chamber for 10–12 days under a 16/8 h photoperiod at a light intensity of 200 μmol m^{-1} s^{-1}, with a day/night temperature of 293/298 K. Plants were collected and the leaves were washed in distilled water and then either analyzed, or alternatively, homogenized in 50 mM Hepes, pH 7.5, and 50 mM $MgCl_2$ and frozen in liquid nitrogen, and stored at −80 °C until used.

TRX-activity test: The insulin–disulphide reduction assay was performed as described previously (Traverso et al., 2007a). Purified TRXs were measured at final concentrations of 2.5 μM in sodium phosphate buffer, 100 mM pH 7.0, 2 mM EDTA, 0.1 mg/ml bovine insulin (Sigma), and 0.25 mM of dithiothreitol (Sigma) as a reductant.

Preparation of the TRX-vinylsulfone silica: Vinylsulfone silica resin was synthesized as described previously (Santoyo-Gonzalez et al., 2007). The synthesis involves two steps. In the first step, activated silica gel (5 g) was added to a solution of [(3-methylamino)-propyl]-triethoxysilane in toluene, and the reaction mixture was heated under reflux for several hours. The solution was then filtered, washed with methanol and dichloromethane, and dried in a vacuum. In a second step, divinylsulfone was added to a suspension of functionalized silica gel obtained in the previous step in 2-propanol/THF (2/1), and the resulting solution was stirred at room temperature for 16 h. The reaction mixture was filtered and washed with methanol and dichloromethane and dried under vacuum to yield the vinylsulfone silica resin.

A 0.75 mg amount of resin with 9 mg of His-tagged protein purified in absence of reductant in 200 mM HEPES pH 7.5 was incubated overnight at room temperature. The reaction was stopped by addition of 565 μL of ethanethiol (90 min at room temperature) and then rinsed with 10 mL of 2 M NaCl and 33 mM DTT to remove unbound protein and excess reagents, and to reduce the bound TRXs. The TRX–vinylsulfone silica was packed into a column and equilibrated in 50 mM HEPES pH 7.6, 50 mM NaCl.

The vinylsulfone silica resin is an attractive support for protein immobilization. It combines the features of the silica (easy handling plus mechanical, chemical, biological and thermal stability) with the high reactivity of the vinylsulfone function towards amine and thiol groups to yield a covalent link. The coupling reaction takes place in aqueous media at pH > 7.5 and 273 K, or at room temperature without any need of previous activation.

Affinity chromatography experiment with TRX-vinylsulfone silica and protein identification: Crude extracts from *P. sativum* plants (10–12 days) were prepared as described above. The column was loaded with 100 mL of crude extract (20 mg/mL) and washed with 50 mM HEPES pH 7.6, 200 mM NaCl, 1 mM $CaCl_2$ to remove nonspecific bound proteins. Bound proteins were eluted with ionic strength (50 mM HEPES pH 7.5, 1.5 M NaCl), and ionic strength plus reducing conditions (50 mM HEPES pH 7.5, 1.5 M NaCl, 20 mM DTT) to distinguish among those proteins that interact with the TRX *via* electrostatic interactions from those that form a disulfide bond.

Three independent experiments for each TRX (*h*1 and *h*2) were analyzed by 2D electrophoresis (data not shown). Tryptic digestion and mass spectrometry were carried out over spots identified in the three replicates by the Central Office for Research Support (SCAI) at Universidad de Córdoba (Spain). The eluted