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Proteomics uncovers proteins interacting electrostatically with thioredoxin in chloroplasts*

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Abstract

The ability of thioredoxin *f* to form an electrostatic (non-covalent) complex, earlier found with fructose-1,6-bisphosphatase, was extended to include 27 previously unrecognized proteins functional in 11 processes of chloroplasts. The proteins were identified by combining thioredoxin *f* affinity chromatography with proteomic analysis using tandem mass spectrometry. The results provide evidence that an association with thioredoxin enables the interacting protein to achieve an optimal conformation, so as to facilitate: (i) the transfer of reducing equivalents from the ferredoxin/ferredoxin–thioredoxin reductase complex to a target protein; (ii) in some cases, to enable the channeling of metabolite substrates; (iii) to function as a subunit in the formation of multienzyme complexes.

Abbreviations: FBPase – fructose-1,6-bisphosphatase; FTR – ferredoxin–thioredoxin reductase

Introduction

The chloroplast ferredoxin/thioredoxin system – composed of ferredoxin, ferredoxin–thioredoxin reductase (FTR) and several thioredoxins (*f*, *m* and the recently added *x* and *y*) – links photosynthetic electron flow to the regulation of a growing number of biochemical processes (Buchanan 1980; Mestres-Ortega and Meyer 1999; Schürmann and Jacquot 2000; Motohashi et al. 2001; Buchanan et al. 2002; Balmer et al. 2003; Lemaire et al. 2003; Marx et al. 2003). The iron–sulfur/disulfide enzyme FTR transfers electrons from ferredoxin to a thioredoxin which, in turn, activates (or deactivates) enzymes and related functional proteins by reduction of a specific disulfide site (Dai et al. 2000; Schürmann 2003). Ideally, this redox cascade would seem to proceed most efficiently if target and possibly other associated proteins formed complexes with members of the system similar to that described for ferredoxin and FTR (Droux et al. 1987; Hirasawa et al. 1988). Such electrostatic interactions would allow for the recognition and optimal orientation of paired proteins for hydrogen transfer and possibly for the channeling of relevant substrates as well.

At present, only one chloroplast enzyme, fructose-1,6-bisphosphatase (FBPase) is known to form an electrostatic (non-covalent) complex with thioredoxin (Soulié et al. 1985; Crawford et al. 1986; Mora-Garcia et al. 1998). The recent identification of new potential thioredoxin targets in chloroplasts raises the possibility that other proteins could form such complexes (Verdoucq et al. 1999; Motohashi et al. 2001; Goyer et al. 2002; Balmer et al. 2003). To pursue this

* Dedicated to Myroslawa Miginiac-Maslow and Peter Schürmann on the occasion of their retirement.

question, we have analyzed chloroplast stroma with affinity chromatography and proteomic techniques. The results suggest that chloroplast thioredoxin (*f*-type) forms an electrostatic complex with a spectrum of stromal components, including target as well as related proteins.

Materials and methods

Preparation of the wild type thioredoxin *f* affinity column, isolation of interacting spinach chloroplast stromal proteins and their separation by 2-D gel electrophoresis and identification by high performance liquid chromatography-tandem mass spectrometry were achieved as previously described (Balmer et al. 2003). The only modifications were that, after loading the affinity column with the stroma extract, (i) a buffer with a lower salt concentration was used to wash the gel (20 mM instead of 50 mM NaCl) and (ii) the proteins interacting with thioredoxin *f* were eluted with buffer containing 200 mM rather than 500 mM NaCl. Electrophoretic analysis revealed that 200 mM NaCl was sufficient and that no new proteins were eluted with 500 mM NaCl.

Results

To gain further insight into associated protein complexes, we bound thioredoxin *f* to an affinity matrix and used a column containing the conjugate to screen chloroplast extract for electrostatically interacting partners. After applying the stromal preparation and washing the affinity column with buffer containing a low salt concentration, interacting proteins were eluted with increased salt, collected, separated by 2-D gel electrophoresis and identified by mass spectrometry. This approach allowed the identification of 28 proteins in the column eluate (Table 1). We confirmed the earlier finding of an association with FBPase and uncovered 27 previously unrecognized partner proteins. The list includes 18 established thioredoxin targets and 10 proteins not earlier known to be associated with thioredoxin. The finding that the present technique with wild type thioredoxin *f* failed to capture other of the additional potential 23 targets isolated earlier with the mutant protein shows that not all covalently interacting targets form stable electrostatic complexes (of the 35 targets isolated by Balmer et al. 2003 and the nine targets isolated by Motohashi et al. 2002, 15 were identified in Table 1). This finding also attests

Table 1. Chloroplast proteins identified that interact electrostatically with wild type thioredoxin *f*. The first number of the peptides column represents the total number of different peptides isolated from the thioredoxin *f* affinity matrix that match the identified protein irrespective of the organism from which it was described. The number in parentheses corresponds to the number of different peptides detected that match a protein from spinach. The molecular mass (kDa) and isoelectric point (pI) of each protein were calculated using the software Compute pI/Mw from the Expasy website (www.expasy.org) with the processed form whenever possible

	Peptides	kDa	pI
New proteins			
Calvin cycle			
GAP dehydrogenase*	14(6)	36	6.7
Rubisco activase*	12(11)	46	5.5
Rubisco small subunit*	7(5)	15	6.3
Rubisco large subunit	17(9)	53	6.1
Phosphoglycerate kinase	11(5)	43	5.3
Transketolase*	3(3)	73	5.7
Translation-related proteins			
28 kDa ribonucloprotein*	2(2)	25	4.4
RNA binding protein 24 kDa	4(4)	23	4.6
RNA binding protein 41 kDa	7(7)	36	5.6
Elongation factor tu*	4(0)	46	5.3
Ribosomal protein S1	3(3)	40	5.0
Ribosomal protein S5	4(4)	34	8.9
Ribosomal protein S30*	2(2)	27	5.9
Ribosomal protein L4	4(4)	27	5.6
Ribosomal protein L21	2(2)	23	6.3
Protein assembly/folding			
70 kDa heat shock protein*	20(11)	65	4.9
Rubisco binding protein*	4(0)	57	4.8
C_4/malate valve			
NADP-malate dehydrogenase*	3(2)	42	5.5
ATP synthesis			
ATP synthase α subunit	4(3)	55	5.2
ATP synthase γ subunit*	7(4)	36	5.8
Nitrogen metabolism			
Glutamine synthetase*	3(0)	39	5.7
Tetrapyrrole biosynthesis			
GSA aminomutase*	2(0)	51	6.4
Fatty acid biosynthesis			
Acetyl-CoA carboxylase*	3(0)	59	7.2
Starch biosynthesis			
ADP-glucose pyrophosphorylase*	2(0)	54	8.9
Protein degradation			
ATP dependent clp protease*	6(0)	102	5.9
HCO_3^-/CO_2 equilibration			
Carbonic anhydrase*	7(3)	24	6.1

Table 1. Continued

	Peptides	kDa	pI
Other			
GTP binding protein	3(0)	45	5.6
Known protein			
Calvin cycle			
Fructose bisphosphatase*	3(1)	39	4.8

The SwissProt ID number for each protein is from the plant with maximal matches: GAP [glyceraldehyde 3-phosphate] dehydrogenase (P19866, P12860); rubisco activase (P10871); rubisco small subunit (Q43832); rubisco large subunit (P00875); phosphoglycerate kinase (P29409); transketolase (O20250); 28 kDa ribonucleoprotein (P28644); RNA binding protein 24 kDa (Q41367); RNA binding protein 41 kDa (O24365); elongation factor tu (Q43467); ribosomal protein S1 (P29344); ribosomal protein S5 (Q9ST69); ribosomal protein S30 (P19954); ribosomal protein L4 (O49937); ribosomal protein L21 (P24613); 70 kDa heat shock protein (Q08080); rubisco binding protein [60 kDa chaperonin subunit α] (P21239); NADP-dependent malate dehydrogenase (P52426); ATP synthase α subunit (P06450); ATP synthase γ subunit (P05435); glutamine synthase (Q9LVI8); GSA aminomutase [glutamate-1-semialdehyde 2,1 aminomutase] (P31593); acetyl-CoA carboxylase (O23960); ADP-glucose pyrophosphorylase [large subunit] (P55243); ATP dependent clp protease [ATP-binding subunit clpa (CD4a)] (P31542); carbonic anhydrase (P16016); GTP binding [like]-protein (Q9SGT3); fructose-1,6-bisphosphatase (P22418).
* Known thioredoxin target (see Balmer et al. 2003 and references therein).

to the specificity of the mutant column in trapping proteins that interact covalently with thioredoxin via the formation of a mixed disulfide. It is noted that, in some cases, the isolated peptides failed to match with spinach peptides owing to the absence of the spinach sequence in the protein database. It should mentioned that, while diagnostic of covalently interacting targets, conserved cysteines are not needed for proteins that interact non-covalently with thioredoxin. Nonetheless, it is pointed out that aside from the 70 kDa heat shock protein and elongation factor Tu, each with a single conserved cysteine, the thioredoxin targets identified in Table 1 have at least 2 conserved cysteines. The possible significance of one versus 2 or more conserved cysteines for covalently interacting thioredoxin targets was raised earlier (Balmer et al. 2003) and is discussed further elsewhere (Balmer et al. 2004).

The proteins identified in the present study function in a spectrum of chloroplast processes (Table 2). In addition to FBPase, four other enzymes of the Calvin cycle, all known targets, showed an electrostatic interaction with thioredoxin – glyceraldehyde 3-phosphate dehydrogenase, rubisco activase, rubisco small subunit and transketolase (Schürmann and Jacquot 2000; Motohashi et al. 2001; Balmer et al. 2003). These enzymes function in the three phases of the Calvin cycle: carboxylation, reduction and regeneration.

The other newly identified proteins found to associate with thioredoxin *f* include known targets functional in a number of chloroplast processes: translation

Table 2. Function of chloroplast proteins that form an electrostatic complex with thioredoxin. Each of the identified processes contains known thioredoxin target proteins as described in the text

Calvin cycle
Carboxylation phase
Rubisco activase
Rubisco
Reduction phase
Phosphoglycerate kinase
GAP dehydrogenase
Regeneration phase
FBPase
Transketolase
Translation-related
RNA stabilization
RNA binding proteins
Translation
Elongation factor tu
Ribosomal proteins
Protein assembly/folding
70 kDa heat shock protein
Rubisco binding protein
C_4/malate valve
NADP–malate dehydrogenase
ATP synthesis
ATP synthase
Nitrogen metabolism
Glutamine synthetase
Biosynthesis
Tetrapyrrole
GSA aminomutase
Fatty acid
Acetyl-CoA carboxylase
Starch
ADP-glucose pyrophosphorylase
Protein degradation
ATP dependent clp protease
HCO_3^-/CO_2 equilibration
Carbonic anhydrase

(28 kDa ribonucleoprotein, elongation factor tu and ribosomal protein S30); protein assembly/folding (70 kDa heat shock protein and rubisco binding protein); C_4/malate valve (malate dehydrogenase); ATP synthesis (ATP synthase gamma subunit); nitrogen metabolism (glutamine synthetase); tetrapyrrole biosynthesis (glutamate-1-semialdehyde 2,1 aminomutase); fatty acid biosynthesis (acetyl-CoA carboxylase); starch biosynthesis (ADP-glucose pyrophosphorylase); protein degradation (ATP dependent clp protease) and HCO_3^-/CO_2 equilibration (carbonic anhydrase) (Schürmann and Jacquot 2000; Balmer et al. 2003).

Ten of the proteins shown in Table 1 were not previously described as covalently interacting targets using mutant thioredoxin affinity columns. Nine of these are known to be members of chloroplast protein complexes in which at least one component is linked to thioredoxin: rubisco large subunit; phosphoglycerate kinase; RNA binding proteins 24 and 41 kDa; ribosomal proteins S1, S5, L4 and L21; and ATP synthase α-subunit. The presence of these proteins in the column eluate may be due to an affinity for one of the target enzymes rather than to a direct interaction with thioredoxin *f* itself. In the Calvin cycle, the rubisco large subunit assumes a quaternary structure with the small subunit in yielding a functional enzyme, and phosphoglycerate kinase forms a complex with glyceraldehyde 3-phosphate dehydrogenase – an association that likely results in substrate channeling (Wang et al. 1996). Similarly, the 24 and 41 kDa RNA binding proteins as well as ribosomal proteins S1, S5, L4 and L21 are known to form complexes with other thioredoxin target proteins – namely, the 28 kDa ribonucleoprotein and ribosomal protein S30, respectively (Hayes et al. 1996; Yamaguchi et al. 2000). Finally, the chloroplast ATP synthase alpha subunit may co-elute with the enzyme's gamma subunit, an established thioredoxin target. Only the tenth component, GTP binding protein is known neither to interact with thioredoxin covalently nor to form a complex with described targets. Accordingly, should this protein not itself form a specific electrostatic complex with thioredoxin, it may have been missed in previous studies or, alternatively, it may associate with a known target for which binding is not evident.

Discussion

Ferredoxin is known to form a strong interaction with different partner proteins to allow efficient electron transfer from PS I to the final acceptor – that is, NADP (ferredoxin–NADP reductase, or FNR), nitrite (nitrite reductase), sulfite (sulfite reductase), α-ketoglutarate (glutamate synthase) and thioredoxin (FTR) (Droux et al. 1987; Dose et al. 1997; Garcia-Sanchez et al. 1997; Akashi et al. 1999). When bound to the thylakoid membrane, such complexes efficiently transfer electrons form PS I to their substrates (Knaff and Hirasawa 1991).

Unlike ferredoxin, the interaction between FTR and thioredoxin does not appear to be driven by charged residues, but rather by hydrophobic interactions (Dai et al. 2000). This weaker link, evident in the salt concentration needed to dissociate the complexes, may allow thioredoxin to be more readily released in the stroma where most of its target proteins are found. To reduce these targets effectively, thioredoxin may require freedom of movement that could be limited if FTR and thioredoxin interacted more strongly via charged residues.

The recognition of thioredoxin by its targets is likely due to complementary structure, thereby making a specific association possible. In this way, thioredoxin is able to identify targets in a large population of stromal proteins and orientated its interacting partner in a complex way that permits efficient dithiol/disulfide exchange. The first examples of electrostatic thioredoxin-protein complexes described were between host thioredoxin and a protein partner from a virus. In *Escherichia coli* infected with the bacteriophage T7, the replication of the T7 DNA is achieved by a polymerase consisting of a 1:1 complex between *E. coli* thioredoxin and phage T7 gene 5 protein (Modrich and Richardson 1975; Mark and Richardson 1976). Surprisingly, even though only reduced thioredoxin can form the complex, polymerase activity is independent of oxidoreductase activity (Adler and Modrich 1983; Huber et al. 1986). In a second case, bacterial thioredoxin forms a complex with several filamentous phage f1 proteins needed for the assembly and export of the virus (Lim et al. 1985). Finally, in a more recent development with mammalian cells, the regulation of apoptosis is believed to be based on a thioredoxin–protein complex. The reduced form of thioredoxin binds to apoptosis signal-regulating kinase 1 (ASK1), thereby preventing the initiation of this process. Upon oxidation of thioredoxin, the complex dissociates and free ASK1 triggers the downstream signaling that leads to cell apoptosis (Saitoh et al. 1998; Arner and Holmgren 2000).

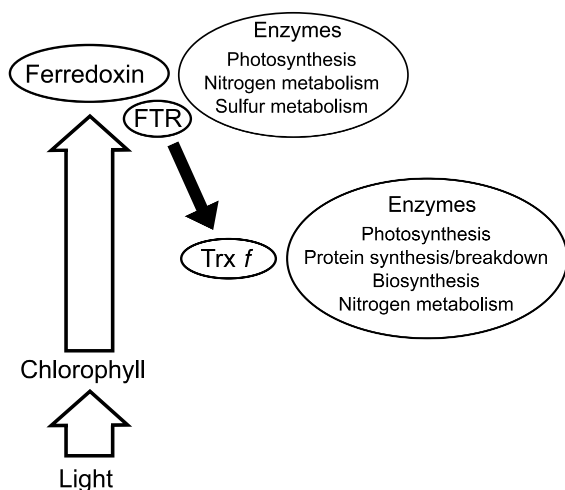

Figure 1. Chloroplast processes whose enzymes form an electrostatic complex with members of the ferredoxin/thioredoxin system.

Prior to the present study, FBPase was the only chloroplast enzyme known to form an electrostatic (non-covalent) complex with a thioredoxin (*f*-type) (Soulié et al. 1985; Crawford et al. 1986; Mora-Garcia et al. 1998). In contrast to the strong interaction observed between ferredoxin and FTR, which requires 200 mM salt for dissociation (Droux et al. 1987; Hirasawa et al. 1988), the complexes with thioredoxin *f* are weaker and are disrupted at 50 mM concentrations. With recognition that the physiological significance of the interactions described awaits further experimentation – perhaps facilitated by recently developed fluorescence resonance energy transfer (FRET) imaging microscopy (Truong and Ikura 2001) – the present results provide evidence that thioredoxin *f* forms an electrostatic complex with a range of other proteins, including participants in photosynthesis as well as other biosynthetic processes of the chloroplast. The complexes formed between members of the ferredoxin/thioredoxin system and interacting proteins appear to assure an efficient transfer of electrons from Photosystem I to destinations that include metabolites (ferredoxin complexes) as well as proteins (thioredoxin complexes) (Figure 1). These complexes may also facilitate metabolite channeling.

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