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SPINACH CHLOROPLAST THIOREDOXINS IN EVOLUTIONARY DRIFT

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SUMMARY: The amino acid sequences surrounding the active sites of spinach chloroplast thioredoxins m and f have been determined. Both types of thioredoxins share common ancester genes with the E. coli one, demonstrated by invariant active site sequences. The m-type thioredoxins have closer homology with the E. coli one in the sequence analyzed as well as in enzymatic specificity, whereas the f-type is less homologous both in sequence and specificity. It suggests that the m-type gene represents a prototype conserved throughout evolutionary processes whereas the f-type has undergone mutations resulting in a modified specificity.

INTRODUCTION: Thioredoxins are ubiquitous small hydrogen carrier proteins participating in a wide variety of biochemical reactions (1). In spinach chloroplasts two types of thioredoxins function in light-activation of enzymes during photosynthesis (2) : the f-type activates fructose 1,6-bisphosphatase and the m-type NADP-dependent malate dehydrogenase (3). E. coli thioredoxin functions as hydrogen donor in the reduction of ribonucleotides, and also constitutes one subunit of T7 DNA polymerase, the other one being the phage gene 5 protein. Chloroplast thioredoxins fully substitute for E. coli thioredoxin in T7 DNA polymerase (4). These facts led us to compare the specificity of the different thioredoxins in the activation of chloroplast enzymes and the sequences important for activation. To this end we present below the amino acid sequence surrounding the active site of the m-type and f-type spinach chloroplast thioredoxins.

MATERIALS AND METHODS: Thioredoxins f and mc purified from spinach according to our published procedure (3) were modified by succinylation and S-carboxymethylation and then cleaved by cyanogen

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bromide. This resulted in two peptides from thioredoxin mc and in three peptides from thioredoxin f. These peptides were separated by chromatography. The C-terminal sequences were determined by carboxypeptidase digestion (5) and the N-terminal sequences by automated Edman degradation (6).

RESULTS AND DISCUSSION: Fig. 1 shows the amino acid sequences surrounding the active site of chloroplast thioredoxins in comparison with the known sequences of thioredoxins from E. coli (7) and Corynebacterium nephridii (8). We have used the redox reactive site to align the different sequences and apply here the numbering of the E. coli protein to all thioredoxins although the four thioredoxins differ in length. It can immediately be seen that the two cysteine residues present in the chloroplast proteins (3) are arranged in the same active site sequence -Cys-Gly-Pro-Cys- as has been demonstrated for other thioredoxins (1,8,9). In all thioredoxin sequences reported to date, except the one of bacteriophage T4 (10), an identical sequence has been found indicating that this stretch of four amino acids has been strictly conserved during evolution. Moreover, the data presented in Fig. 1 suggest that the conserved active site sequence of thioredoxins still includes Trp in position 31 and Lys in position 36, to the left and to the right of the redox reactive S-S bridge resulting in an active site sequence of -Trp-Cys-Gly-Pro-Cys-Lys-.

Thioredoxin mc shows more striking homologies with thioredoxins of E. coli or C. nephridii between the residues 24 and 53, with 21 of the 30 residues being the same as in E. coli thioredoxin.

Apart from the active site, the sequence of thioredoxin f is quite distinct from the others. Two of the three Met are near the region of the active site, at positions 27 and 53. From the other residues only positions 26 and 50 are the same as in E. coli thioredoxin, adding up to a homology of only 26% between chloroplast thioredoxin f and E. coli thioredoxin in the region of the active site. The Val at position 24 in thioredoxin f is also found at the corresponding position in C. nephridii thioredoxin.

These sequence differences are reflected in the pronounced specificity when activating chloroplast enzymes with different thioredoxins. It has already been known that chloroplast fructose 1,6-bisphosphatase is activated efficiently only by reduced thioredoxin f, whereas NADP-dependent malate dehydrogenase can be activated by thioredoxin m as well as by thioredoxin f, although with different kinetics (3). In view of the similarities between thioredoxin

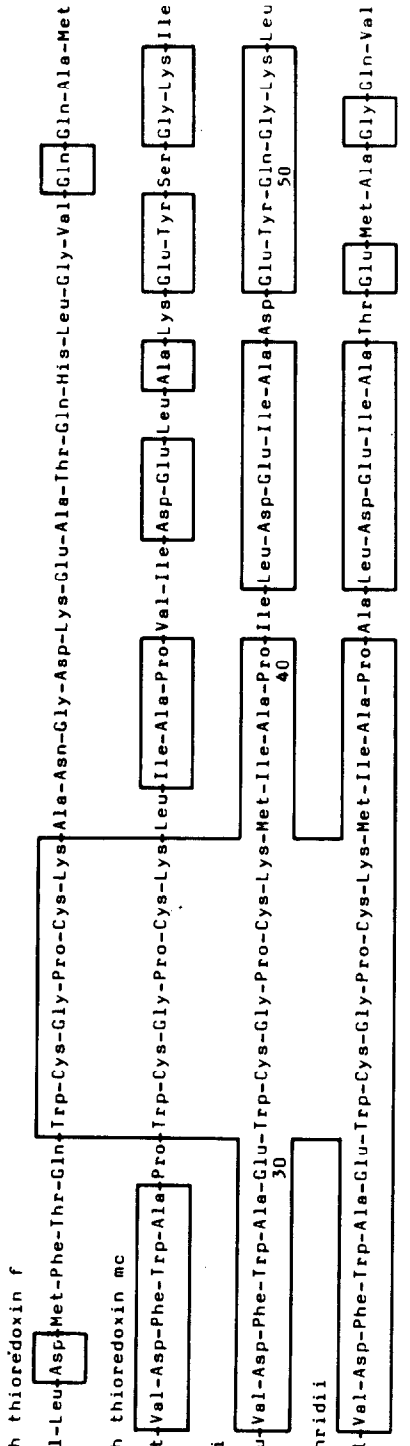

Fig. 1 : Comparison of active site sequences of thioredoxins. Identical residues are enclosed in frames.

TABLE 1 : EFFECTIVENESS OF DITHIOTHREITOL-REDUCED THIOREDOXINS IN ACTIVATION OF FRUCTOSE 1,6-BISPHOSPHATASE

	Activity at pH 7.9, 1.5 mM Mg; measured after activation by thioredoxin							Activity at pH 8.5, 16 mM Mg^{++}; no thioredoxin activation
Thioredoxin conc.	1 µM				10 µM			
Thioredoxin	-	mb	E. coli	f	mb	E. coli	f	
Activation time, min at 25°C								
5	0	0	0	76	0.4	0.4	117.7	120.2
30	0.7	0.7	0.7	116.4	2.4	2.4	94.5	120.7
60	1.7	1.7	1.3	123.7	5.8	6.3	122.4	123.7

Activation mixture (total volume 100 µl) : Tris-HCl pH 7.9 10 µmol; dithiothreitol, 0.5 µmol; fructose 1,6-bisphosphatase 13.6 µg; thioredoxin as indicated.

Reaction mixture (total volume 1 ml) : Tris-HCl pH 7.9 resp. 8.5, 100 µmol; $MgSO_4$, 1.5 resp. 16.0 µmol; EGTA, 0.1 µmol; fructose 1,6-bisphosphate, 1.0 µmol; NADP, 0.3 µmol; yeast glucose-6-phosphate dehydrogenase, 0.7 units; phosphoglucose isomerase, 1.75 units. Activated enzyme, 5 µl.

Activities are expressed in units / mg enzyme.

The last column shows the maximal activities displayed by the enzyme when tested under the indicated in vitro conditions.

mc and E. coli thioredoxin and their differences to thioredoxin f we have reexamined their efficiency in activating the chloroplast enzymes.

The results in Table 1 confirm that only thioredoxin f is capable of activating pure chloroplast fructose 1,6-bisphosphatase which reaches full activity within short time at low thioredoxin concentration. Neither thioredoxin mb (or mc) nor E. coli thioredoxin can significantly activate this enzyme under our experimental conditions. Similar results have also been obtained with thioredoxin from C. nephridii. In contrast, all thioredoxins tested activate NADP-dependent malate dehydrogenase (Fig. 2). This enzyme is almost fully activated after 15 minutes in presence of 2 µmolar reduced thioredoxin regardless which type of thioredoxin is used. Thioredoxin mc (or mb) is however the most efficient activator. Experiments using the complete light dependent activation system (11) have given similar results indicating that thioredoxin from E. coli can also be efficiently reduced by the ferredoxin-thioredoxin reductase as has already been observed (12).

Our results indicate that in the region of the protrusion (13) carrying the redox reactive S-S bridge of the protein, m-type thioredoxins

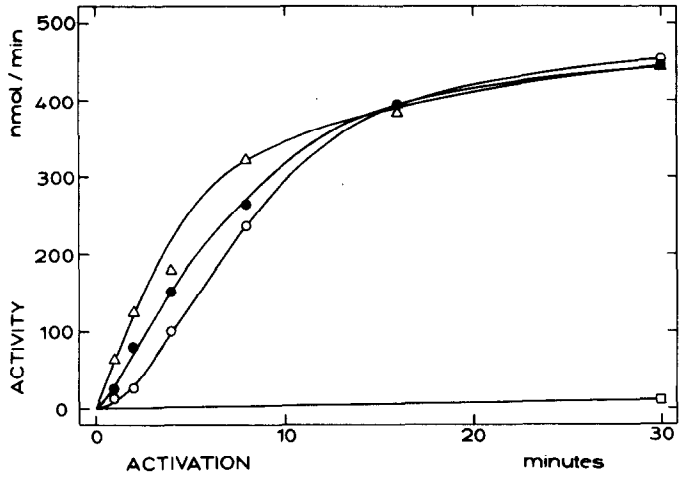

Fig. 2 : Time - Dependent Activation of NADP-Malate Dehydrogenase by Dithiothreitol - Reduced Thioredoxins.
7 µg of enzyme were incubated in 200 µl containing 20 µmol Tris/HCl buffer pH 7.9, 1 µmol dithiothreitol and 0.4 µmol thioredoxin at 25°C. The activation mixtures contained spinach thioredoxin mb (△——△), spinach thioredoxin f (○——○), thioredoxin from E. coli (●——●) or no thioredoxin (□——□). At different times 5 µl samples were withdrawn and injected into 1 ml reaction mixtures containing 100 µmol Tris/HCl buffer pH 7.9, 0.5 µmol oxaloacetate and 0.2 µmol NADPH. Changes in absorbance at 340 nm were followed spectrophotometrically.

from spinach are quite similar to the bacterial thioredoxins from E. coli and C. nephridii with over 60% homology. However the overall amino acid composition of thioredoxin m (3) suggests that other regions of this protein must be more different from bacterial thioredoxins. Spinach m-type thioredoxin and bacterial thioredoxins can be used interchangeably to activate NADP-dependent malate dehydrogenase. On the other hand, fructose 1,6-bisphosphatase can only be activated by thioredoxin f. Since the active site sequence of thioredoxin f is the same as in thioredoxin m, i.e. -Trp-Cys-Gly-Pro-Cys-Lys-, but the adjacent sequences are quite different from the other thioredoxins tested, we may postulate that the flanking sequences are crucial in the specific protein-protein interaction needed for enzyme activation. To substantiate this hypothesis further work on the complete amino acid sequence of the spinach thioredoxins is under way.

Fully active T7 DNA polymerase was obtained when instead of the usual E. coli thioredoxin the chloroplast thioredoxin m or even f are added to the phage gene 5 protein. It was reported that one third of the C-terminal part of E. coli thioredoxin is involved in the protein-protein

interaction with phage gene 5 protein to bring about polymerase activity (14). Preliminary sequence analysis of the corresponding C-terminal region of thioredoxin mc revealed little homology with the E. coli thioredoxin. We may, therefore, conclude that the phage gene 5 protein interacts with protein domains of variant amino acid sequences.

Since thioredoxins are found in all phyla they represent an interesting case to study evolution of a gene or a gene family of a hydrogen carrier protein. The most conservative part of the protein is the active site, and as shown in this paper, it consists of exactly the same hexapeptide as was reported for two bacterial thioredoxins. From other studies we know (9,1) that thioredoxins from phylogenetically distant organisms like anabaena, yeast and calf have the tetrapeptide -Cys-Gly-Pro-Cys- in common. The additional homology in the sequence between bacterial and plant m-type thioredoxin strongly suggests that this type represents a prototype protein in the family of thioredoxins which may be involved in a variety of functions as was partly shown in the present study and may play a most important role throughout the phylogenetic tree. The importance of this prototype protein kept its gene conservative during evolution. The other, f-type gene may be duplicated from the prototype gene and have undergone quite a few mutations except for the active site encoding region. This resulted in a different specificity maintaining at the same time its redox function and the protein-protein interaction capability with T7 phage gene 5 protein.

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REFERENCES:

1. Holmgren, A. (1981) Trends Biochem. Sci. 6, 26-28.
2. Buchanan, B.B., Wolosiuk, R.A. and Schürmann, P. (1979) Trends Biochem. Sci. 4, 93-96.
3. Schürmann, P., Maeda, K., Tsugita, A. (1981) Eur. J. Biochem. 116, 37-45.
4. Harth, G., Geider, K., Schürmann, P. and Tsugita, A. (1981) FEBS Lett. 136, 37-40.
5. Tsugita, A., Gregor, I., Kubota, I., v.d. Broek, R. (1979) Cytochrome Oxidase (King, T.E. et al., eds), pp. 67-77, Elsevier Biomedical Press, Amsterdam.
6. Tsugita, A., Blazy, B., Takahashi, M., Baudras, B. (1982) FEBS Lett. 144, 304-307.

7. Holmgren, A. (1968) Eur. J. Biochem. 6, 475-484.
8. Meng, M., Hogenkamp, H.P.C. (1981) J. Biol. Chem. 256, 9174-9182.
9. Gleason, F.K., Holmgren, A. (1981) J. Biol. Chem. 256, 8306-8309.
10. Sjöberg, B.M. and Holmgren, A. (1972) J. Biol. Chem. 247, 8063-8068.
11. Schürmann, P., Jacquot, J.P. (1979) Biochim. Biophys. Acta 569, 309-312.
12. Wolosiuk, R.A. and Buchanan, B.B. (1977) Nature 266, 565-567.
13. Holmgren, A., Söderberg, B.O., Eklund, H. and Bränden, C.I. (1975) Proc. Natl. Acad. Sci. 72, 2305-2309.
14. Holmgren, A., Kallis, G.B. and Nordström, B. (1981) J. Biol. Chem. 256, 3118-3124.