Published in FEBS Letters 36, issue 1, 37-40, 1981
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THIOREDOXINS FROM SPINACH CHLOROPLASTS SUPPLEMENT PHAGE T7 GENE 5 PROTEIN FOR DNA POLYMERASE ACTIVITY

Günter HARTH, Klaus GEIDER, Peter SCHÜRMANN+ and Akira TSUGITA*

*Max-Planck-Institut für Medizinische Forschung, Abteilung Molekulare Biologie, Jahnstr. 29, 6900 Heidelberg, *European Molecular Biology Laboratory, Meyerhofstr. 1, 6900 Heidelberg, FRG* and *+Laboratoire de Physiologie végétale et Biochimie, Université de Neuchâtel, Neuchâtel, Switzerland*

1. Introduction

Thioredoxins are ubiquitous low M_r hydrogen carrier proteins. This class of proteins has first been described as a hydrogen donor for the ribonucleotide reductase in *Escherichia coli* [1]. It has since been shown that thioredoxins have various functions in the cellular metabolism [2]. Their function suggests also evolutionary conservation, but they differ in size and in amino acid sequence, when isolated from various sources. In the chloroplasts of green plants thioredoxins are part of a light-dependent activation mechanism for some key enzymes [3]. In the light, electrons from chlorophyll reduce ferredoxin and the reduced ferredoxin transfers its electrons via the enzyme ferredoxin–thioredoxin reductase to thioredoxin which then activates certain enzymes like fructose 1,5-bisphosphatase and NADP-dependent malate dehydrogenase. The thioredoxins from spinach chloroplasts have been purified and resolved into 3 different proteins: thioredoxin *f* (11 100 M_r), capable of activating chloroplast fructose 1,5-bisphosphatase and malate dehydrogenase, thioredoxin *mc* (10 700 M_r) and thioredoxin *mb* (10 500 M_r), both capable of activating only malate dehydrogenase. These thioredoxins have been characterized by amino acid compositions and N-terminal and C-terminal sequences. The results allowed us to conclude that thioredoxin *f* is a protein different from the *m*-type thioredoxins, whereas thioredoxin *mb* and *mc* are N-terminal redundant isomers [4]. Thioredoxin-S_2 (11 700 M_r) from *E. coli* is one of the best characterized thioredoxins [5]. The complete sequence and the 2.8 Å three-dimensional structure are known and show the presence of the catalytically active cystine S–S bridge. The size and amino acid composition differs from the 3 spinach thioredoxins.

The DNA polymerase induced by phage T7 consists of 2 subunits, an 84 000 M_r protein encoded by the phage gene 5 and a 12 000 M_r protein, which is the thioredoxin of the *E. coli* cell [6]. This protein complex could be separated into its subunits and subsequently reconstituted [7]. Phage T7 gene 5 protein could be supplemented in vitro with *E. coli* thioredoxin to yield fully active T7 DNA polymerase [8]. The purified gene 5 protein still exhibits enzymatic activities as the 3′–5′ hydrolysis of single-stranded DNA [9]. Hydrolysis of duplex DNA, however, requires as the DNA polymerase activity the presence of thioredoxin. For replication of duplex DNA, T7 DNA polymerase interacts with the phage-encoded gene 4 protein [10]. This enzyme system hydrolyses nucleoside triphosphates and unwinds nicked duplex DNA. It has been used to replicate specifically cleaved phage fd RF [11].

It was of interest to see whether thioredoxins from spinach chloroplasts whose prime function is assumed to be the activation of photosynthetic enzymes could substitute for the *E. coli* thioredoxin in the T7 DNA polymerase and whether this system distinguishes between the 3 thioredoxins.

2. Materials and methods

Phage T7 DNA polymerase and phage T7 gene 4 protein were purified as in [11]. Phage T7 gene 5 protein was isolated as in [12]. The thioredoxins *mb*, *mc* and *f* were isolated from spinach chloroplasts as in [4]. *E. coli* thioredoxin was generously provided by Dr Arne Holmgren, Stockholm.

T7 gene 5 protein was assayed for complementation of DNA polymerase activity and additionally for DNA unwinding activity in the presence of T7 gene 4 protein as follows:

(A) The incubation mixture contained T7 gene 5 protein (0.03 unit) and 3 μg denatured T7-DNA in 20 μl of a buffer containing 88 mM K-phosphate (pH 7.6)/6.7 mM Mg^{2+}/5 mM 2-mercaptoethanol and deoxyribonucleoside triphosphates (100 μM each) with the label as [^{3}H]TTP (2700 cpm/pmol). Incubation was for 20 min at 30°C. The acid-insoluble radioactivity was determined. The enzyme units are defined as in [11].

(B) The incubation mixture contained T7 gene 5 protein (0.03 unit, 0.1 μg), T7 gene 4 protein (0.2 unit) and phage fd RF II_0 (50 ng), which is fd RF specifically nicked by fd gene 2 protein [13]. The 20 μl assay further contained the buffer and nucleotides listed above. The specific radioactivity of [^{3}H]TTP was 2700 cpm/pmol. Incubation was also for 20 min at 30°C.

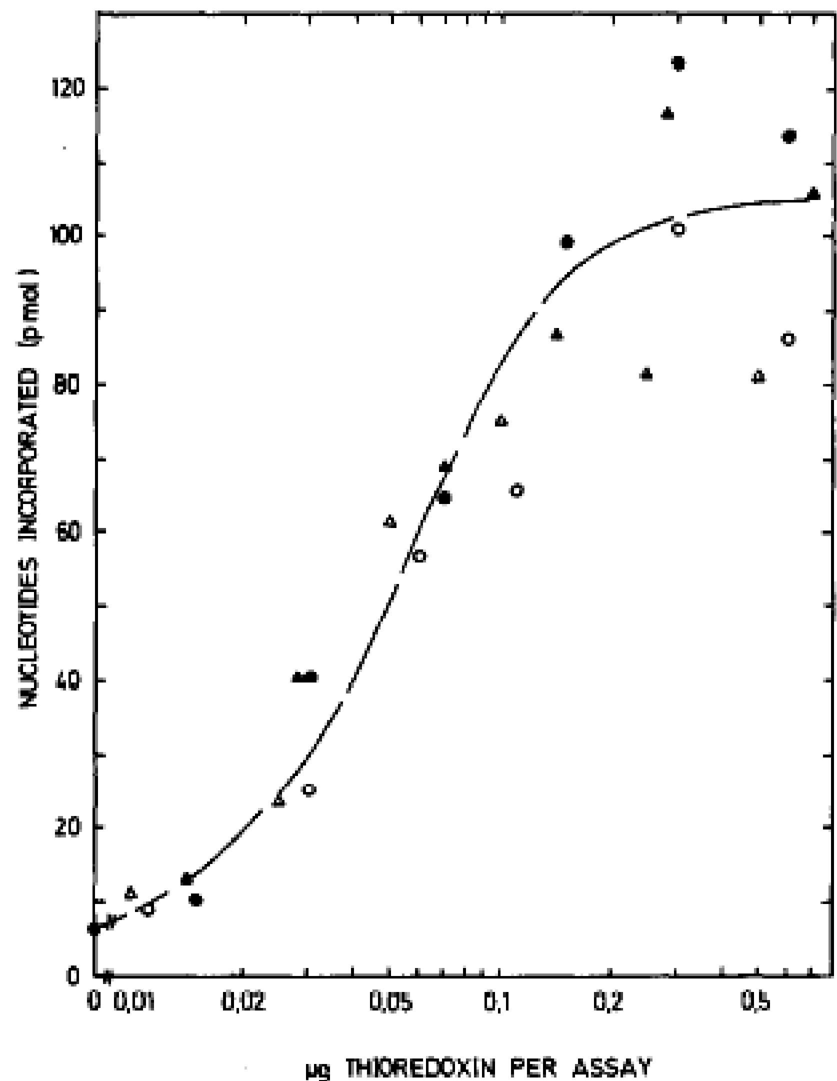

Fig.1. Complementation of the large subunit of T7 DNA polymerase by various thioredoxins. Template DNA was denatured T7 DNA. Replication was performed with T7 gene 5 protein as in section 2. The thioredoxins added were from *E. coli* (•) and from spinach chloroplasts: *mb* (○), *mc* (▲) and *f* (△).

3. Results

To reconstitute phage T7 gene 5 protein for its DNA polymerase activity, the purified protein was supplemented with thioredoxin from *E. coli*. The template DNA was either denatured T7 DNA or denatured calf thymus DNA (fig.1). The looped back ends of these DNAs served as primers for initiation of DNA synthesis. At saturating thioredoxin concentrations DNA synthesis was at least 10-fold above the nucleotide incorporation obtained with T7 gene 5 protein alone. Saturation was achieved at 0.3 μg *E. coli* thioredoxin for 0.1 μg T7 gene 5 protein. Thioredoxins from spinach chloroplasts complemented T7 gene 5 protein as well as did the *E. coli* thioredoxin. This was not only found for the amount of stimulation but also for the response to the concentration of the various thioredoxins. We therefore conclude that total replacement of *E. coli* thioredoxin by the spinach proteins has occurred.

Complementation of T7 gene 5 protein was also measured in a more complex reaction which also requires T7 gene 4 protein for DNA synthesis (table 1). The template was either denatured DNA or phage fd RF specifically cleaved with fd gene 2 protein [13]. The assay with gene 4 protein and nicked fd RF was optimized with purified T7 DNA polymerase [11].

T7 gene 5 protein had only a minute effect on DNA synthesis using single-stranded DNA as template, and the reconstituted T7 DNA polymerase could barely incorporate nucleotides into the nicked fd RF (table 1). DNA synthesis on the nicked duplex DNA was, however, greatly stimulated when T7 gene 5 protein was not only supplemented with one of the thioredoxin species, but also with T7 gene 4 protein. Only slight differences were observed for the various types of thioredoxins applied. This indicates that the reconstituted T7 DNA polymerase does not show a discriminatory effect for its concerted reaction together with gene 4 protein in respect of the thioredoxin used for supplementation.

Table 1
Complementation of the large subunit of T7 DNA polymerase by various thioredoxins

Template	Denatured T7-DNA	Denatured calf thymus DNA	Phage fd $RFII_0$
	[DNA synthesized (pmol)][a]		
(A) Thioredoxin			
None	7	6	0.9
E. coli	124	130	2.3
mb	102	81	1.8
mc	118	81	1.9
f	82	74	2.3
(B) Thioredoxin			
None	8	6	1.2
E. coli	179	184	9.2
mb	97	118	8.8
mc	162	70	9.0
f	145	102	8.5

a The incubation conditions were as in section 2; thioredoxin was added to 0.3 μg/20 μl assay, when indicated
(A) Complementation of the large subunit of T7 DNA polymerase
(B) Complementation of the large subunit of T7 DNA polymerase in the presence of T7 gene 4 protein

4. Discussion

The structural divergence of thioredoxins could indicate specialized functions of the proteins as in the activation of chloroplast enzymes. Isolation and amino acid analysis revealed 3 distinct thioredoxins in spinach chloroplasts. 'Composition divergence' calculation shows no correlation between *E. coli* thioredoxin and any of the spinach thioredoxins, whereas definite close relation was shown between spinach type *f* and type *m* [14]. Recent sequencing of the thioredoxin *mc* revealed that the preparation of *mc* contains ~25% of another terminal redundant isomeric protein, *md*, which lacks the amino-terminal alanine residue of *mc* (K. Maeda, A. T., P. S., unpublished). The separation of *mc* and *md* has not yet been achieved. The terminal sequences were different between the *E. coli* protein and spinach *m*, whereas spinach *f* has a blocked amino-terminus. Thioredoxins from yeast and calf liver contain the same invariant S–S active center as *E. coli* thioredoxin [2]. Spinach thioredoxins also contain 2 cysteine residues suggesting the existence of similar sequences in the active centers. The total acidic amino acid residues and contents of basic amino acids are found to be in quite good agreement throughout all thioredoxins. One-third of the total residues from the C-terminus of *E. coli* thioredoxin contains a region involved in protein–protein interactions [15]. This region may be highly conserved in the chloroplast thioredoxins making them all compatible as subunits of T7 DNA polymerase. The intimate contact of *E. coli* thioredoxin with phage T7 gene 5 protein implicates a sensitive tool to match other thioredoxins for the ability to replace the *E. coli* thioredoxin. The data given here show that all 3 proteins can efficiently replace *E. coli* thioredoxin for the restoration of T7 DNA polymerase activity. They could furthermore fully complement in the strand-unwinding reaction displayed by T7 DNA polymerase together with T7 gene 4 protein. These results could be interpreted to mean that thioredoxin from other sources than *E. coli* are still largely conserved in essential parts of their amino acid sequence or in an active area to allow complementation of the T7 gene 5 protein. To better understand the interaction between the large subunit of T7 DNA polymerase and the spinach chloroplast thioredoxins the primary structure of these proteins has to be determined.

Acknowledgement

This research was aided by grant 3.452.79 from the Swiss National Science Foundation to P. S.

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