Further evidence for a role of sulfhydryls in the thioredoxin dependent activation of corn NADP-malate dehydrogenase

Use of a cysteine free mutant of *E. coli* thioredoxin

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The kinetics of activation and reduction of the corn leaf NADP-malate dehydrogenase reveal that plant thioredoxins play a dual role in the activation of this enzyme in the presence of dithiothreitol (e.g. reductive and conformational). A mutant thioredoxin of *E. coli* in which Cys_{32} and Cys_{35} are replaced by an Ala and a Ser residue, respectively, was tested in the activation of corn NADP-malate dehydrogenase either in the presence of DTT or in a light reconstituted system. While native *E. coli* or plant $thioredoxin_m$ strongly activated the NADP-malate dehydrogenase, the mutant thioredoxin was unable to promote any activation of the enzyme. At the same time the mutant thioredoxin stimulated the activity of T_7 DNA polymerase. These results clearly confirm that the thioredoxin requirement observed in the activation of NADP-malate dehydrogenase is primarily needed for redox reactions and not for structural/conformational effects as is the case for the T_7 DNA polymerase assay.

Mutant *Thioredoxin* *Malate dehydrogenase (NADP$^+$)* *Redox reaction* *Light regulation*

1. INTRODUCTION

In higher plant chloroplasts the activity of several enzymes is regulated by light [1,2]. Among the mechanisms proposed as responsible for the light regulation, the best documented by far is the ferredoxin-thioredoxin system. In this system the target proteins are regulated by reduction through a series of reactions involving the following components: chloroplastic electron transport, ferredoxin, ferredoxin-thioredoxin reductase and enzyme specific thioredoxins [3,4]. In vitro, the light system can be replaced by an artificial reductant, dithiothreitol (DTT) and the thioredoxins [5,6]. While it is clear that a target enzyme like for example the NADP-malate dehydrogenase (NADP-MDH; EC 1.1.1.82; malate dehydrogenase (NADP$^+$)) is reduced by such a system [7], the role of thioredoxin itself in the activation sequence of this enzyme can be disputed. Of course the most likely role for thioredoxin in this sequence would be to function as a disulfide oxidoreductase as is well known for several other reactions in which this protein is involved [8,9]. This hypothesis is very likely since DTT shows an exceptional reactivity with the disulfide of *E. coli* thioredoxin [10], a protein that differs only mildly from spinach $thioredoxin_m$ [11]. Thus in the system composed of DTT, $thioredoxin_m$ and NADP-MDH it seems likely that the thioredoxin molecule would be reduced first and that in turn the reduced thioredoxin molecule would be able to activate the NADP-MDH by reducing a disulfide bridge on each subunit of this enzyme as proposed in [7]. One could however postulate an alternative hypothesis in which thioredoxins in this reaction would not serve primarily as a reductant but rather as a structural/conformational factor, thus enhancing a direct reduction of NADP-MDH by DTT or by the ferredoxin-thioredoxin reductase in the

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physiological light system. Indeed a structural role was found for thioredoxin in the stimulation of the T_7 DNA polymerase. This was first suggested by the absence of requirement for DTT in this reaction [12,13]; it was confirmed by using mutants of the *E. coli* thioredoxin protein that did not contain any disulfide bridge [14]. Since plant thioredoxins can be replaced by *E. coli* thioredoxin in the DTT or light activation of NADP-MDH [15,16], it was possible to use mutants of this protein that were lacking the functional disulfide bridge in order to test the hypothesis formulated above. We report in this paper the effect of one of these mutant proteins on the activation of corn leaf NADP-malate dehydrogenase.

2. MATERIALS AND METHODS

Spinach leaves were purchased at a local market. Procedures used to grow corn and pea plants were as published [16,17].

Authentic thioredoxins (e.g. those that possess a disulfide bridge) were assayed by their capacity to activate the NADP-MDH either in the presence of DTT or with the light activation system. The mutant *E. coli* thioredoxin was assayed through its function as a subunit of the T_7 DNA polymerase that induces the catalytic activity of this enzyme.

The procedure used for the purification of corn NADP-MDH was similar to the one described in [17] except for the use of a chromatography on Matrex Red (Pharmacia) as described in [18] as a final step. Ferredoxin, thioredoxin$_m$ and ferredoxin-thioredoxin reductase were purified from spinach leaves as described in [5,19]. All the protein components of the ferredoxin-thioredoxin system were homogeneous when analysed by SDS-polyacrylamide gel electrophoresis. Pea thylakoids were purified and stabilized as described in [20]. The light activation reconstituted system was obtained by mixing in appropriate concentrations the following components: thylakoids, ferredoxin, ferredoxin-thioredoxin reductase, thioredoxin$_m$ and NADP-MDH. When indicated thioredoxin$_m$ was replaced by the mutant thioredoxin of *E. coli*. DTT-thioredoxin dependent activation of NADP-MDH was carried out as described in [5]. The activity of corn NADP-MDH was assayed in the direction of the synthesis of malate as described in [5]. Further experimental details are given in the legends of the figures.

The quantitation of thiol groups on the NADP-MDH was realized by carboxymethylating the protein with [^{14}C]iodoacetate. The protein was first incubated in the presence of 2 mM DTT and thioredoxins when indicated; then radioactive sodium iodoacetate (2 μCi/μmol) was added in a slight excess (1 mM) and incubated with the protein for 15 min in the dark at 20°C. The radioactive protein was separated from the free radioactive label by precipitation with 10% trichloroacetic acid (final concentration); the pellets were washed 3 times and the radioactivity of the protein was measured after dissolution with 2% SDS in the buffer 100 mM Tris(HCl), pH 8.9.

T_7 DNA polymerase was assayed as described in [12] by mixing the gene 5 protein (3 mM) with varying concentrations of thioredoxins and denatured calf thymus DNA. The incorporation of [^{3}H]dNTP into the acid insoluble material was measured as in [12]. The *E. coli* mutant thioredoxin was a gift from Dr P. Model.

The chemicals used were of the highest quality available and usually from Sigma except for NADPH and oxaloacetate that were from Boehringer.

3. RESULTS AND DISCUSSION

The Ala-Ser mutant of *E. coli* thioredoxin was tested in the DTT dependent activation of corn leaf NADP-MDH. As is apparent in table 1 the mutant protein did not show any activity in this test even when used at very high concentrations (260-fold higher than *E. coli* thioredoxin) while native spinach thioredoxin$_m$ or native *E. coli* thioredoxin were equally efficient in this reaction. In the absence of a reductant, neither spinach thioredoxin nor *E. coli* thioredoxin were able to promote any activation of the NADP-MDH. The only observed effect for the mutant protein was to slightly increase the rate of activation when added together with native thioredoxin$_m$, DTT and the NADP-MDH (fig.1).

The mutant thioredoxin was also tested in the light activation of NADP-MDH. It appears (fig.2) that while native thioredoxins promoted a good activation of the NADP-MDH, the mutant protein was totally unable to activate the enzyme.

Table 1

Effect of different concentrations of the Ala-Ser mutant thioredoxin on the DTT dependent activation of corn leaf NADP-MDH

	Time of preincubation (min):		10	20
			NADP-MDH activity (units/mg NADP-MDH)	
2 mM DTT	No TRX (control)	2	2	
	Native TRX_m or *E. coli* TRX	10.8 μM	139	286
		0.38 μM	100	200
		0.17 μM	33	106
	TRX Ala, Ser	12.5 μM	3	2.5
		45 μM	2	2.5
No DTT	Native TRX_m	10 μM	0	0
	MDH alone		0	0

The activation conditions were identical to those described in fig.1. Time of activation was 15 min

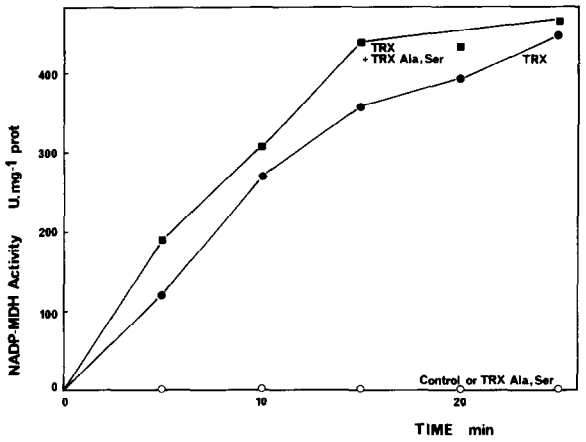

Fig.1. Effect of the mutant Ala-Ser thioredoxin in the DTT dependent activation of corn leaf NADP-MDH: time course. The activation conditions were as follows: 100 μl of the activation medium contained 20 μmol Tris-HCl, pH 8.0, 0.5 μmol DTT, 4 μg NADP-MDH (0.5 μM); native spinach $thioredoxin_m$ or *E. coli* thioredoxin (TRX) was present in the range 0.17–10 μM while *E. coli* mutant thioredoxin (TRX Ala, Ser) was 45 μM. After 10–20 min incubation at 20°C, aliquots of 20 μl were used for the determination of the NADP-MDH activity as in [5].

The mutant thioredoxin used in these experiments was not denatured by the lyophilisations/transports needed for these experiments since it was found to behave identically to untreated mutant thioredoxin in the T_7 DNA polymerase assay (fig.3). The effect of increasing concentrations of the mutant Ala_{32}, Ser_{35} *E. coli* thioredoxin in the T_7 DNA polymerase assay is shown in this figure. As observed earlier [13], the mutant protein is effective in stimulating DNA synthesis by T_7 DNA polymerase although the mutation decreases its affinity for the gene 5 protein by about 100 times when compared to the native *E. coli* thioredoxin. This is indicative that the mutation induced a conformational change of the thioredoxin. In its altered conformation the thioredoxin was still able to stimulate the activity of DNA polymerase, but not the activation of NADP-MDH. There are two ways of explaining the behavior of the mutant thioredoxin in the DTT dependent activation of NADP-MDH: (i) native thioredoxin does not play a conformational role in the DTT activation of NADP-MDH, and hence mutant thioredoxin does not play this role either; (ii) the mutation affected the conformational effects of thioredoxin more drastically in the NADP-

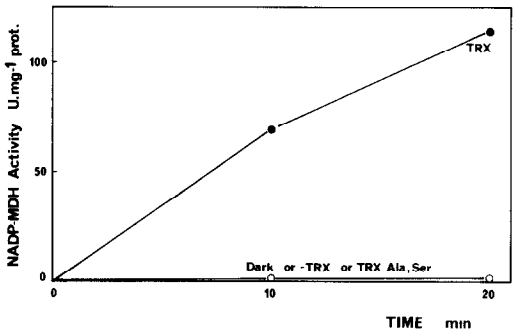

Fig.2. Light activation of NADP-MDH in the presence of the Ala-Ser thioredoxin mutant. The activation conditions were as follows: the incubation medium contained in 100 μl of 10 μmol Tris-HCl, pH 8.0, 20 μg catalase, 10 μg ferredoxin (8 μM), 4 μg ferredoxin-thioredoxin reductase (1 μM), 12 μg NADP-MDH (1.5 μM), chloroplast thylakoids (equivalent to 26 μg chlorophyll) and *E. coli* or spinach thioredoxins (TRX) 10 μM (native) or 45 μM (mutant). The activation was carried out under N_2 at 20°C under constant illumination (300 W/m^2). At the indicated times, aliquots were removed with a syringe and used for the determination of the NADP-MDH activity.

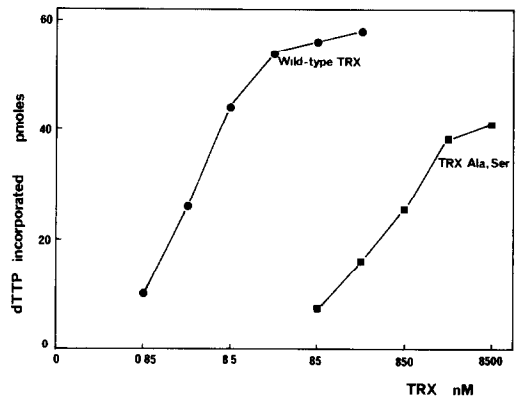

Fig.3. Stimulation of T_7 DNA polymerase by mutant thioredoxin. Gene 5 protein (3 nM) and thioredoxin of varying concentrations were incubated with denatured calf thymus DNA at 30°C, pH 7.5. After 20 min, the incorporation of ^{3}H]dNTP was recorded as in [12].

MDH activation than in the DNA polymerase test.

In order to test if thioredoxin also plays a conformational role in the dithiol dependent NADP-MDH activation, we performed the following experiment: the kinetics of the NADP-MDH reduction and activation were followed in the presence of DTT alone (fig.4A) or of DTT and thioredoxin (fig.4B). At pH 7.9 and 20°C, 2 mM DTT was absolutely inefficient in activating the enzyme; at the same time the enzyme was only partially reduced (0.6 SH appeared per mol of subunit) (fig.4A). When thioredoxin was added the enzyme was activated with the obtention of a plateau of maximal activity after about 30 min incubation. In the same conditions, the enzyme was reduced at a rate which was slightly faster than the one observed for the activation (fig.4B). In these conditions the enzyme was fully reduced with an SH content of 2 thiols

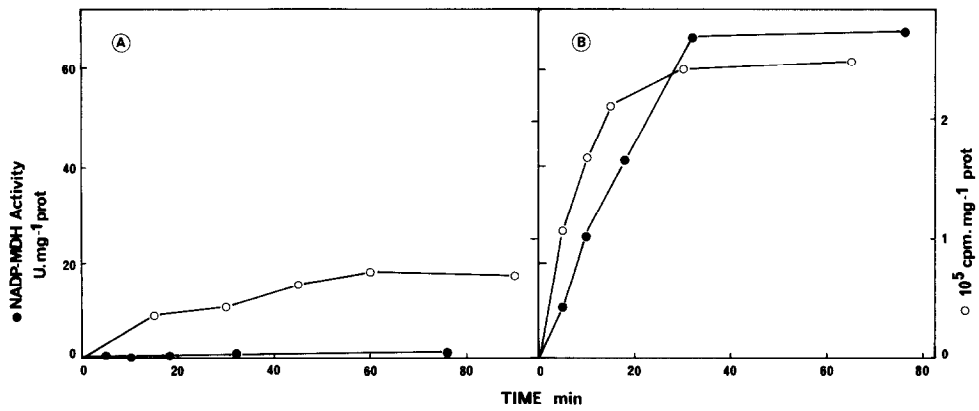

Fig.4. Kinetics of the activation and reduction of NADP-MDH by dithiothreitol (A) or dithiothreitol and spinach thioredoxins (B). The incubation conditions were as follows: pH 7.9, DTT concentration 2 mM, MDH concentration 1.1 μM, and thioredoxin concentration 8 μM (B). The number of thiols appearing on the NADP-MDH were determined after substracting the radioactivity incorporated in the thioredoxins as determined in a separate experiment.

per monomer of NADP-MDH [21]. These results lead to the following observations: (i) since DTT alone can partially reduce the enzyme but not activate it, it is likely that thioredoxins in addition to their putative redox role should also play a conformational role in the activation; (ii) the lag observed between the reduction of the NADP-MDH and the appearance of its activity (fig.4B) is most likely related to a slow conformational change needed after reduction of the protein in order to reach its active conformation.

In conclusion, these results indicate that the thioredoxins play a dual role in the activation of the NADP-MDH. Their primary function is to reduce the enzyme and this reduction is followed by a slower conformational change essential for the appearance of the catalytic activity. The conformational change itself seems to be dependent on the structure of the thioredoxin molecule as indicated by the experiments performed with the mutant thioredoxin. Such a conformational change was postulated earlier for the pea leaf NADP-MDH, based on experiments performed without any reductants (DTT or reduced thioredoxin) where a guanidine-HCl dependent activation of the enzyme was observed [18]. This could not be reproduced for the corn or the spinach (not shown) NADP-MDHs but the conformational effect is no doubt present. For the corn enzyme however, clearly the conformational change must be preceded by a reduction of the enzyme.

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