THIOREDOXIN$_m$ IN PEA CHLOROPLASTS: CONCENTRATION AND REDOX STATE UNDER LIGHT AND DARK CONDITIONS

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1. Introduction

Thioredoxins have been characterized as small, heat-stable proteins which have multiple functions as redox carriers in various bacterial, plant and mammalian systems [1]. For *Escherichia coli* thioredoxin a change in the redox state due to the dithiol/disulfide group in the active center has been shown [2]. Thioredoxin can be reduced either by dithiothreitol (DTT) or enzymatically. In plants they are involved in the modulation of several enzymes [3]; reduced via photosynthetic electron flow by ferredoxin–thioredoxin-reductase. When this flow stops, thioredoxin is oxidized by a yet unknown oxidant. Both the reduced and the oxidized form specifically mediate the activation or inactivation of chloroplast enzymes, as shown for NADP-dependent malate dehydrogenase and glucose 6-P-dehydrogenase [4]. Several thioredoxins in plant systems have been described which appear to differ in M_r-value and specificity with respect to the modulated enzymes [5,6]. Spinach chloroplasts contain 3 thioredoxin isomers with distinct isoelectric points [7]. The molecular mechanism of the modulation of chloroplast enzymes has not yet been elucidated. One possibility is a reduction of the enzymes as in the case of insulin, where thioredoxin acts as a catalytic hydrogen carrier [8]. Another mode of action is the formation of a stable complex with the enzyme as in a phage-induced DNA-polymerase of *E. coli* [9].

In the chloroplast system it is important to note that light- or dark-modulation apparently reaches completion in terms of seconds [10]. In a first attempt to elucidate the type of mechanism operative in chloroplasts, a quantification of thioredoxin was undertaken and the changes in its redox state during light and dark were followed.

Abbreviations: MDH, NADP-dependent malate dehydrogenase; Td$_m$, thioredoxin$_m$; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin

2. Materials and methods

Intact chloroplasts were isolated from 3-week-old pea plants (*Pisum sativum* L. var. Kleine Rheinländerin). For the extraction the chloroplasts were washed twice with the isotonic resuspension medium and then ruptured osmotically with hypotonic buffer [4]. Modulatable MDH was partially purified by ammonium sulfate fractionation and Sephycryl S-200 gel filtration [4]. Thioredoxin$_m$ was prepared as in [6]. Anti-Td$_m$-serum was obtained using Td$_m$ from this preparation. Preimmune serum was taken from the same rabbit. In each case the γ-globulin fraction obtained by ammonium sulfate precipitation (0–40% saturation) and resuspension in 0.2 M citrate buffer (pH 6.5) was used.

The standard incubation mixture for the determination of Td$_m$ activity consisted of non-limiting amounts of partially purified MDH, 50 mM Hepes–KOH (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 10 mM KCl, 0.1% BSA, 10 mM DTT and the Td$_m$-sample (final vol. 100 μl). After 5 min incubation at 26°C MDH-activity was assayed immediately. The MDH assay mixture (1 ml final vol.) consisted of 100 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM oxaloacetate and 0.2 mM NADPH in addition to the incubated enzyme solution. The change of absorbance at 340 nm was monitored with a CARY 219 spectrophotometer. Chlorophyll concentration was determined according to [11]. Protein content was estimated as in [12].

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3. Results

3.1. *Quantification of total Td_m in the stroma of isolated intact pea chloroplasts*

3.1.1. Direct titration

The effect of DTT-reduced thioredoxin on the activation of MDH shows a linear dependency on the thioredoxin concentration, provided that non-limiting amounts of enzyme are present. This fact is represented by the calibration curve for purified Td_m (fig.1). Thus unknown amounts of Td_m in the chloroplast stroma fraction could be determined from the activation effect in the standard incubation mixture. To this end endogenous MDH was precipitated from the stroma by heat treatment (2 min; 80°C) and removed by subsequent centrifugation. The supernatant was then assayed for its Td_m content. From several experiments 0.1 mM stromal Td_m was calculated.

3.1.2. Immunotitration

Td_m activity was inhibited by addition of anti-Td_m-serum raised against purified pea chloroplasts Td_m in a rabbit. The antibody titer was determined by the inhibitory effect of an aliquot volume of antiserum on the activation of MDH by known amounts of Td_m in a standard incubation mixture containing the antiserum instead of BSA. The activity obtained in the presence of the same amount of pre-immune serum was taken as a control for each assayed Td_m-concentration. Fig.2 shows a typical titration curve for 20 µl antiserum, which was sufficient to inhibit 0.65 µg

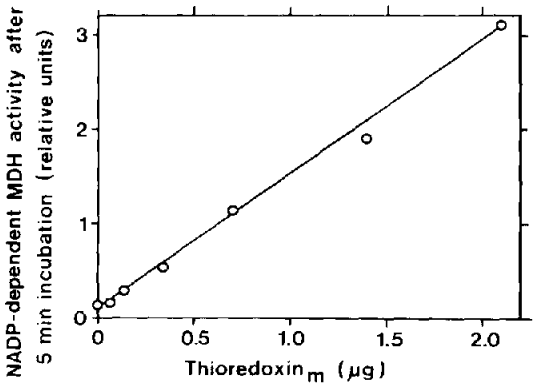

Fig.1. Activation rate of MDH as influenced by the concentration of purified and DTT-reduced Td_m.

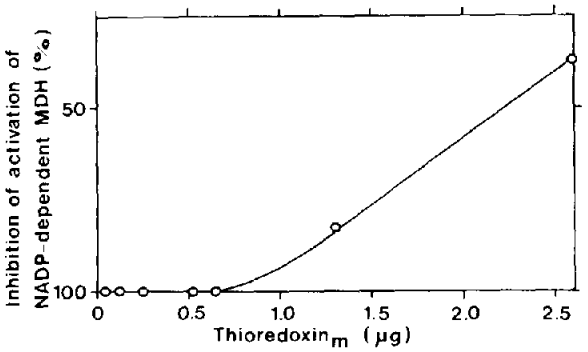

Fig.2. Activity of MDH with increasing amounts of purified and DTT-reduced Td_m in the presence of 20 µl anti-Td_m-serum.

Td_m under the given conditions (5 min; 26°C). With the calibrated antiserum, unknown amounts of Td_m in chloroplast extracts could be determined by the same titration procedure. From such an experiment, 0.1 mM Td_m in the stroma was determined. A similar result (0.16 mM Td_m) was obtained with antiserum against *E. coli* thioredoxin (a generous gift from A. Holmgren, Stockholm), which has been shown to inhibit the modulation of chloroplastic MDH [4].

3.1.3. Td_m concentration.

The data obtained with both quantification methods are in agreement. On the basis of 30 µl stroma/mg chl. [13], 3–5 mM Td_m/mg chl. was calculated.

3.2. *Quantification of reduced Td_m under light and dark conditions*

NEM irreversibly affects only the reduced molecules of MDH and of its activating factors by alkylation of the sulfhydryl groups. The oxidized portion can then be reduced in a subsequent illumination period or by DTT-treatment [14]. This fact was utilized for the determination of the redox state of Td_m in illuminated and darkened chloroplasts. Intact illuminated or darkened or broken and then DTT-treated chloroplasts were mixed with a 4-fold vol. of 50 mM phosphate buffer (pH 8.0) containing 20 mM NEM. After 15 min mercaptoethanol was added (final conc. 100 mM) to capture unreacted reagent. The chloroplast membranes were removed by centrifugation. The supernatant was heated for 2 min (80°C) and denatured protein (including endogenous MDH) was separated from the heat-stable Td_m by centrifugation. The Td_m concentration was then

Table 1
Equilibrium levels of reduced Td_m in darkened, illuminated and DTT-treated chloroplasts

Chloroplasts	Treatment	Reduced Td_m [% of total]
Intact	Dark	8–30
Intact	Light	62–77
Broken	DTT	80

The chloroplast suspensions (4 mg chl/ml) were treated as shown in fig.3 for 20 min in the light and dark, respectively, or DTT was added to a 2-fold diluted sample at 5 mM final conc.

determined as in section 3.1.1. Since the reduced Td_m (dithiol) had been inactivated by NEM, only the oxidized portion could be determined in the standard activation assay with DTT. The total amount of Td_m (reduced and oxidized) was obtained from an aliquot volume of the same chloroplast preparation. The percentage of reduced Td_m was calculated from the difference between total and oxidized Td_m (table 1).

3.3. *Change of redox-state of Td_m and of the MDH activity upon light/dark/light transitions*

In order to test for a correlation between the redox state of Td_m and the actual activity of the modulated enzyme, both parameters were followed upon light/dark/light transitions.

Oxidized Td_m was estimated in samples taken as indicated in fig.3. Simultaneously, parallel samples were withdrawn, diluted 10-fold with ice-cold bidistilled water and spun in a microfuge to remove the membrane fraction. An aliquot volume of the supernatant was then assayed immediately for MDH-activity. The results are shown in fig.3.

As can usually be observed, the first illumination period after the isolation of the plastids leads to a slower and less complete activation of MDH than do following ones. Thus the second illumination period as shown in fig.3 should represent typical behavior upon dark/light transition. However, in both casses a constant level of reduced Td_m (total minus oxidized) is attained within a few minutes. Upon darkening decrease of enzyme activity and increase of the percentage of oxidized thioredoxin occur at the same rate.

4. Discussion

The thioredoxin concentration in *E. coli* cells and calf liver, the only systems studied thus far in this respect, in 15 μM [15,16]. Td_m at 100–160 μM in

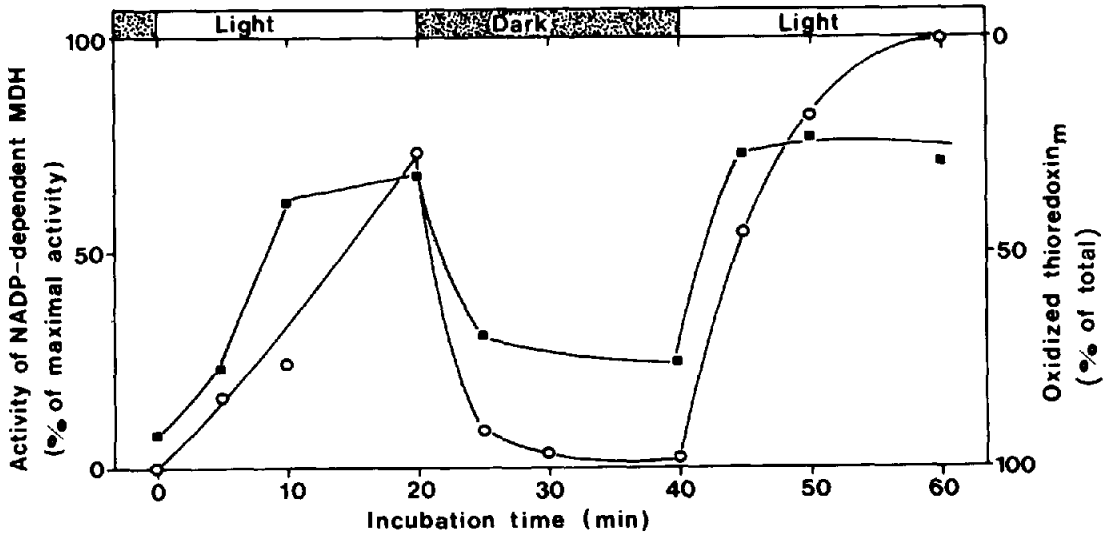

Fig.3. Time-course of MDH activity (○) and of the level of oxidized Td_m (■) in isolated intact chloroplasts upon light/dark/light transitions; chlorophyll 3.6 mg/ml; total Td_m was 2.82 nmol/mg chl. which corresponds to 94 μM Td_m in the stroma; 100% activity corresponded to 71 μmol oxaloacetate reduced/mg chl. and h.

chloroplasts is even higher and is comparable to photosynthetic substrate levels, which range from 80 μM (ribulose 1,5-bisphosphate) to 800 μM (fructose 1,6-bisphosphate) [17]. Since a small portion of Td_m appears to be membrane bound, the actual concentration might be somewhat higher [18].

In [19] a partial association of MDH and its activator (presumably Td_m) with the thylakoids was shown. A specific binding or association of Td_f and chloroplast fructose 1,6-bisphosphatase has been demonstrated in [20]. In view of this, the high concentrations of Td_m suggest the formation of a complex between the modulatable enzyme and thioredoxin rather than pointing to a catalytic hydrogen transfer by the latter.

The results in fig.3 indicate that MDH activity further doubles, after the pool oxidized Td_m has already attained a constant level. There are two explanations for this phenomenon:

(i) The constant level of reduced Td_m does not indicate a static, but rather a steady state due to a constant turnover of Td_m by regeneration of reduced Td_m subsequent to its consumption in the modulation reaction.

(ii) The total Td is reduced in one rapid step. Activation of the enzyme is brought about by a subsequent and slower reaction in which a stable interaction between reduced Td_m and the modulatable enzyme is established.

The first possibility seems less likely from the interpretation of the high Td_m concentration. In addition, the activation rate and the maximal activity of MDH in a reconstituted chloroplast system are dependent on the amount of Td_m which is either reduced by DTT [19] or by light-driven electron flow (unpublished). Furthermore, dark modulation of MDH and glucose 6-P-dehydrogenase could not be completed with limiting amounts of oxidized thioredoxin [4]. Therefore a certain ratio of Td_m to enzyme molecules appears to be obligatory for rapid and complete modulation of enzyme activity*. To establish such a stoichiometry, the concentration of MDH in the chloroplast stroma has to be evaluated.

* On the other hand, it cannot be ruled out that the need for high thioredoxin concentrations in the in vitro experiments was due to the disruption of the in vivo topology of molecules involved upon dilution

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