Molecular properties and thioredoxin-mediated activation of spinach chloroplastic NADP-malate dehydrogenase

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1. INTRODUCTION

There is no doubt that light-generated reducing equivalents can be used in C_3 plants to perform a number of reactions occurring outside the chloroplasts [1–3]. Since the chloroplastic membrane is impermeable to NADPH and to reduced ferredoxin, it is currently believed that these reduction reactions in the cytoplasm involve the so-called C_4-dicarboxylic acid shuttle [4]. A specific malate translocator located on the chloroplastic membrane exports, outside the chloroplast, this dicarboxylic acid which is used in the cytosol to perform a number of reduction reactions [3]. NADPH formed during photosynthesis is used to reduce oxaloacetate to malate in the chloroplastic stroma. Chloroplastic malate dehydrogenase plays therefore an important role in the generation of reducing power used in reactions occurring in the cytoplasm.

Moreover, crude extracts containing chloroplastic malate dehydrogenase are totally inactive in the dark and become active upon illumination. The effect of light is mimicked by a mixture of dithiothreitol and thioredoxin [5,6]. Surprisingly, chloroplastic NADP-malate dehydrogenase has never been purified to homogeneity. Therefore, nothing is known as to its structure, its reaction mechanism and the molecular bases of photoregulation by thioredoxin. Moreover one totally ignores whether several *iso*-forms of malate dehydrogenase exist in the chloroplast.

The aim of this paper is to describe a purification procedure of chloroplastic NADP-malate dehydrogenase, to report some molecular properties of this enzyme as well as some features of its activation by dithiothreitol and thioredoxin.

2. MATERIALS AND METHODS

Spinach leaves were purchased from a local market. NADPH, oxaloacetate, $NADP^+$, dithiothreitol, glucose 6-phosphate dehydrogenase and fructose 1,6-bisphosphate were purchased from Boehringer, ovalbumin and bovine serum albumin from Fluka, α-chymotrypsinogen from Sigma. The matrix gel (red A) was obtained from Amicon and 2′,5′-ADP–Sepharose 4B from Pharmacia. Other standard reagents were of the highest grade commercially available. Fructose 1,6-bisphosphatase was isolated and purified from spinach chloroplasts as in [7]. Thioredoxin *m* was purified from spinach chloroplasts according to a new technique (submitted). Chloroplasts were prepared following [8].

M_r-Value determinations were effected by molecular sieving over Sephadex G-200 and by sedimentation equilibrium. Columns were calibrated with protein of known M_r (fructose 1,6-bisphosphatase 160 000, glucose 6-phosphate dehydrogenase 110 000, bovine serum albumin 68 000, ovalbumin 42 000, α-chymotrypsinogen 26 500). Analytical centrifugation experiments were performed with a Beckman model E ultracentrifuge equipped with an interferometric system for M_r determination and with UV absorption optics for sedimentation constant estimation. Most of the absorbance measurements were made with an Acta M VII spectrophotometer. Slab gels electrophoresis under denaturing conditions was done as in

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[9]. Protein used as standards were glucose 6-phosphate dehydrogenase, bovine serum albumin, fructose 1,6-bisphosphatase and α-chymotrypsinogen

Estimations of malate dehydrogenase activity were carried out by monitoring, with a Gilford spectrophotometer, the absorbance decrease of NADPH in presence of oxaloacetate. Malate dehydrogenase is incubated 30 min at 30°C with 10 mM dithiothreitol in 100 mM phosphate buffer (pH 8). A unit of malate dehydrogenase activity was defined as the amount of enzyme required to consume 1 μmol substrate/min under conditions of maximum rate, when the ratio dithiothreitol/enzyme is 1.6×10^6.

3. RESULTS

Spinach leaves (3 kg) were crushed in a Waring blendor in the presence of 3 l 50 mM (pH 7.5) Tris–HCl buffer and 1 mM EDTA. Temperature was kept to 2°C. Cellular debris were discarded by centrifugation and filtration. The crude extract was then acidified to pH 4.8 with formic acid and the resulting pellet discarded by centrifugation. After adjusting the pH of the supernatant to 7 with NaOH, the extract was fractionated with solid ammonium sulphate. The precipitate obtained between 30–55% saturation was collected by centrifugation and suspended in 250 ml 5 mM phosphate buffer (pH 7). The resulting extract was dialyzed 3 times for 24 h against the same buffer, and submitted to DEAE-cellulose chromatography (DE 23 SS Serva). The column equilibrated with 10 mM phosphate buffer (pH 7) was eluted with a linear gradient of pH 7 phosphate buffer from 0.02–0.15 M. If an extract from isolated chloroplasts is submitted to the same type of chromatography, the same elution profile of NADP-malate dehydrogenase activity is obtained. This enzyme is therefore chloroplastic. Moreover, NAD-malate dehydrogenase activity, which is very high in leaf extracts, is very low in chloroplast extracts. Fractions containing NADP-malate dehydrogenase were collected, concentrated on Diaflo (PM 10), dialyzed against 20 mM phosphate buffer (pH 7), 1 mM EDTA, and submitted to affinity chromatography on matrix gel (ligand: procion red HE 3B). Elution was carried out with a linear gradient of KCl (fig.1A). Fractions eluted

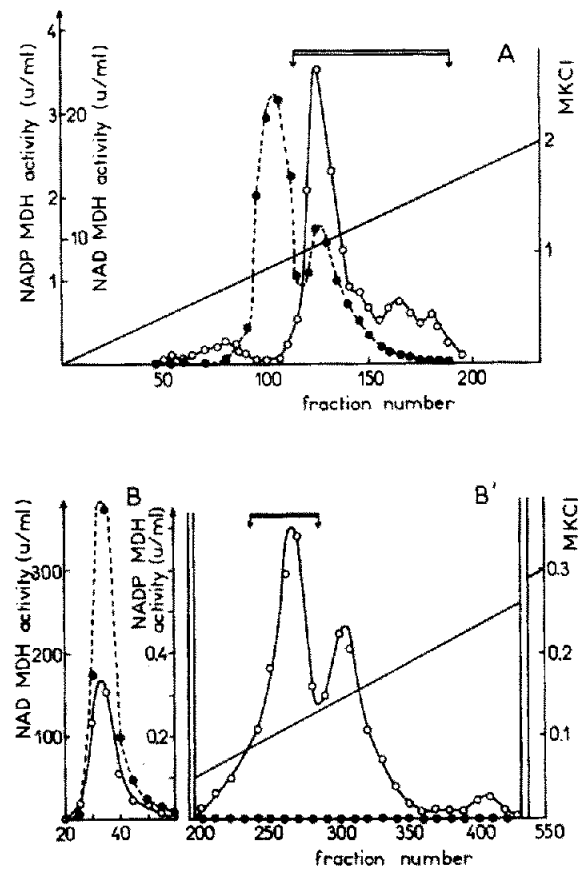

Fig.1. Affinity chromatography of NADP-malate dehydrogenase.

(A) Matrix gel affinity chromatography. The column (15.5 × 2.6 cm) is equilibrated in a 20 mM phosphate buffer (pH 7) and then washed with the same buffer. Elution is performed with a gradient of KCl (0–2 M) in the same buffer. Fractions (1.7 ml) are collected and tested for their NAD (•---•) and NADP-malate dehydrogenase (○——○) activities. Fractions located between the two arrows are collected and submitted to 2′,5′-ADP–Sepharose chromatography.

(B,B′) 2′,5′-ADP–Sepharose chromatography. The column (16 × 1.6 cm) is equilibrated with 100 mM phosphate buffer (pH 7) plus 5 mM NAF and 5 mM EDTA, then washed with the same phosphate buffer (B) and eluted with a linear gradient of KCl (0–0.3 M (B′)). Fractions (0.7 ml) are collected and tested for their NAD-malate dehydrogenase (•---•) and NADP-malate dehydrogenase activities (○——○). Fractions located between the 2 arrows are collected and submitted to various tests of homogeneity; MDH = malate dehydrogenase.

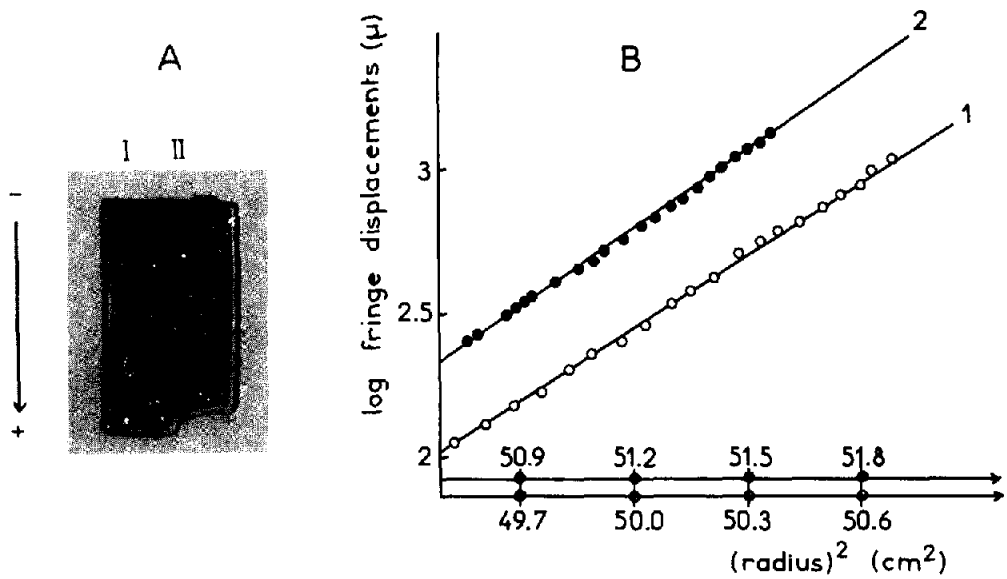

Fig.2. Homogeneity and M_r estimation of chloroplastic NADP-malate dehydrogenase. (A) Slab gel electrophoresis (non-denaturing conditions): (I) 6 μg protein; (II) 12 μg protein. (B) M_r estimation of native and denatured NADP-malate dehydrogenase: (1) Equilibrium centrifugation under non-denaturing conditions – 1 mg enzyme/ml, 25°C, 24 000 rev./min; (2) equilibrium centrifugation under denaturing conditions – protein denatured by 6 M guanidinium chloride (pH 8) and 30 mM β-mercaptoethanol, 25°C, 40 000 rev./min.

above 1 M of KCl were collected, concentrated and dialyzed. The extract thus obtained was submitted to another affinity chromatography on 2′,5′-ADP–Sepharose. On washing the column, a first peak of activity was obtained (fig.1B). If a linear gradient of KCl was applied to the column, 3 subsequent peaks of activity were observed. Fractions corresponding to the first of these peaks were collected and appear to be free of any protein contamination as shown below. The yield of the purification procedure is shown in table 1.

The enzyme preparation thus obtained is homogeneous as determined by 10% polyacrylamide slab gel electrophoresis (fig.2A) and analytical centrifugation. The protein has an apparent sedimentation constant of 4.7 S. Molecular sieving through a Sephadex G-200 column gave 56 000 ± 3000 M_r (fig.3A). On equilibrium sedimentation, M_r was 55 680 ± 1100 (fig.2A). If the enzyme has been denatured by 2% sodium dodecylsulfate and 30 mM β-mercaptoethanol, electrophoresis on polyacrylamide slab gels calibrated by different protein standards gave M_r 28 500 ± 3000 for the malate dehydrogenase subunit (fig.3B). This result may be confirmed by equilibrium sedimentation of the enzyme, denatured by 6 M guanidinium chloride (pH 8) and 30 mM β-mercaptoethanol (fig.2A). The M_r obtained under these conditions

Table 1

Purification of a chloroplastic NADP-malate dehydrogenase from spinach leaves (3 kg)

Fraction	Protein (mg)	Activity (U)	Spec. act. (U/mg protein)
Extract	16 600	300	0.018
DEAE	1 071	215	0.20
Matrix	34	150	4.4
Sepharose	0.54[a]	25[a]	46[a]

[a]Isoenzyme studied here; other values refer to a mixture of isoenzymes

Details of the purification procedure are given in the text. Activation of the enzyme is carried out with dithiothreitol alone used at a concentration that gives maximum activation

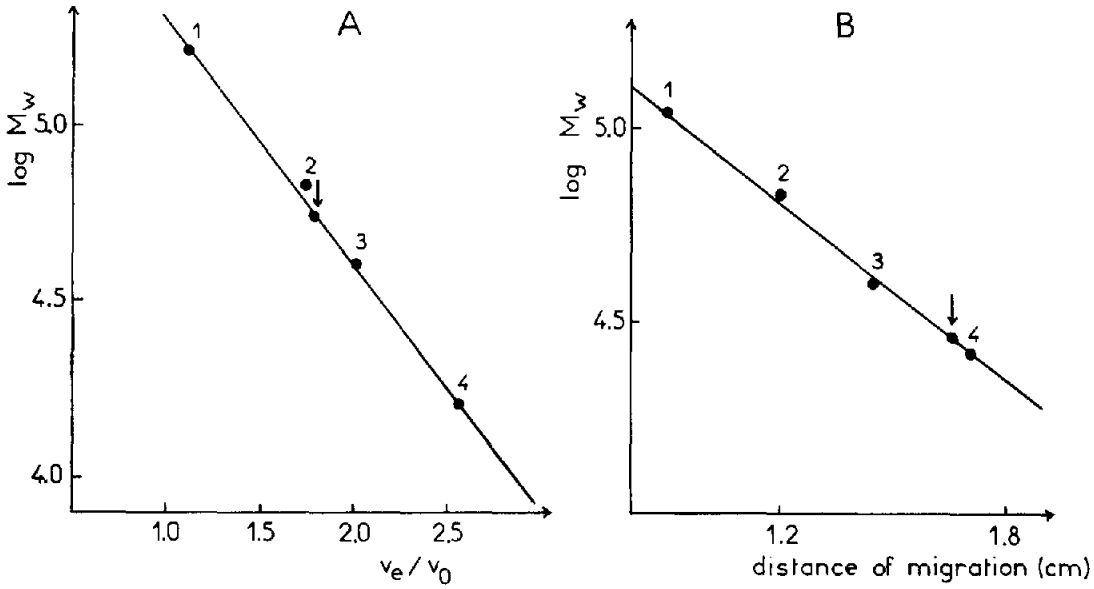

Fig.3. M_r estimation of NADP-malate dehydrogenase by molecular sieving and analytical gel electrophoresis. (A) Molecular sieve chromatography on Sephadex G-200: The column (95 × 1.6 cm) is equilibrated with 5 × 10^{-3} M phosphate buffer (pH 7) containing NaCl (final conc. 0.1 M). Exclusion volume of the column (V_0), is determined with dextran blue. Points marked with numbers 1–4 correspond to the elution ratios (V_e/V_0) of the various proteins used as standards (from 1–4: fructose-bisphosphatase tetramer, bovine serum albumin, ovalbumin, α-chymotrypsinogen). The point marked with an arrow shows the elution ratio of NADP-malate dehydrogenase and corresponds to M_r 56 000 ± 3000. (B) Analytical gel electrophoresis under denaturing conditions. After denaturation of chloroplastic malate dehydrogenase by 2% SDS and 30 mM β-mercaptoethanol, 6 μg enzyme was submitted to electrophoresis on slab gels calibrated with different protein standards. Points marked 1–4 correspond to the distance of migration of the various markers (from 1–4: glucose 6-phosphate dehydrogenase, bovine serum albumin, fructose bisphosphatase monomer, α-chymotrypsinogen, respectively). The point marked with an arrow shows the migration of the polypeptide chain of chloroplastic NADP-malate dehydrogenase and corresponds to M_r 28 500 ± 3000.

is 28 400 ± 600. These results suggest that the protein is made up of 2 apparently identical subunits.

Pure chloroplastic NADP-malate dehydrogenase is inactive if dithiothreitol has been omitted from the reaction mixture. Dithiothreitol alone slightly activates pure NADP-malate dehydrogenase (fig.4A). However, thioredoxin *m* markedly increases both the rate of enzyme activation and the maximum level of enzyme activity. Full activation may be obtained in the presence of thioredoxin *m* in ~10 min, which is compatible with light activation in vivo [10]. When dithiothreitol alone is used to activate the enzyme, the extent of activation declines as a function of incubation time of the enzyme with dithiothreitol (fig.4B). The incubation time that gives maximum activity depends on the ratio dithiothreitol/enzyme. If dithiothreitol is rapidly removed by molecular filtration from a mixture of dithiothreitol plus enzyme, activity is no longer detectable (fig.4B). Similar results are obtained on removing thioredoxin and dithiothreitol from a reaction mixture containing NADP-malate dehydrogenase and these 2 reactants (fig.4A).

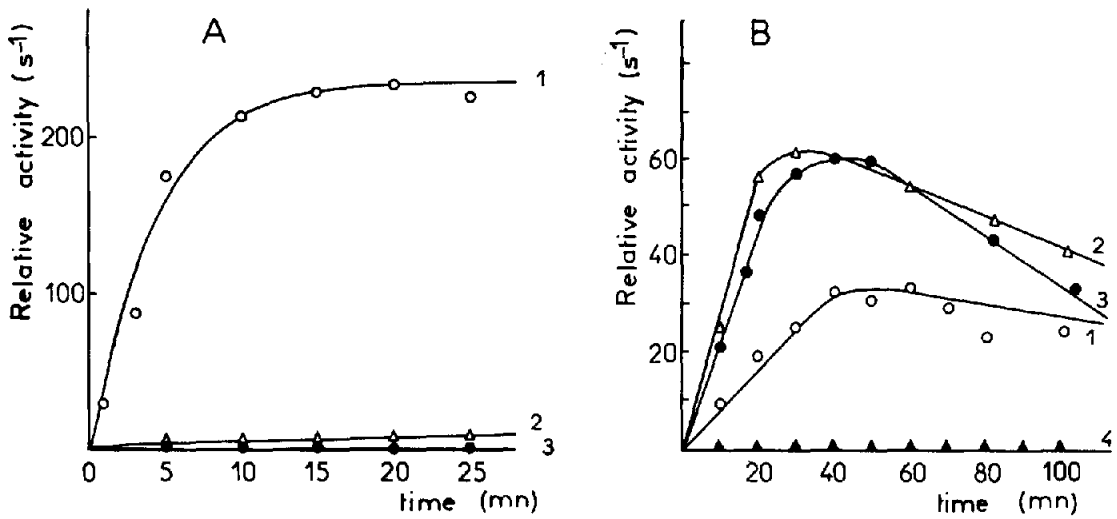

Fig.4. Activation of pure NADP-malate dehydrogenase by dithiothreitol and thioredoxin *m*. (A) Activation by thioredoxin *m* and dithiothreitol of pure NADP-malate dehydrogenase: (1) The enzyme (0.23 M) is incubated with thioredoxin *m* (14 μM) and dithiothreitol (5.7 mM) for the times indicated in abscissa; The enzyme activity is then measured on an aliquot of the incubation mixture; (2) thioredoxin has been omitted; other conditions as above; (3) the enzyme incubated 30 min with thioredoxin *m* and dithiothreitol is passed over a Sephadex G-25 column in order to remove excess dithiothreitol and thioredoxin. The enzyme is collected and its activity is tested. (B) Activation of NADP-malate dehydrogenase by dithiothreitol: Thioredoxin *m* has not been introduced in the reaction mixture. Inactive malate dehydrogenase is incubated with dithiothreitol for various times and for various dithiothreitol/enzyme ratios. In curves 1–3 the ratio is equal to 880 000, 1 600 000 and 3 300 000, respectively. Curve (4): after 30 min incubation of enzyme with dithiothreitol, the reductant is removed as in fig. 4A. The activity is tested. In all experimental conditions NADPH and oxaloacetate concentrations are 0.4 mM and 3 mM, respectively. Relative activity, means activity/enzyme concentration.

4. DISCUSSION

Several forms of NADP-malate dehydrogenase exist in spinach chloroplasts. One of them has been purified to homogeneity and appears to be made up of 2 apparently identical polypeptide chains.

If removing dithiothreitol from the reaction mixture mimicks the effect of removing the light, it is reasonable to speculate that upon a light–dark transition NADP-malate dehydrogenase becomes deactivated, and therefore the export of reducing power from chloroplast to cytosol ceases. The spontaneous deactivation of NADP-malate dehydrogenase after removal of dithiothreitol and thioredoxin is at variance with what has been shown to occur with chloroplastic fructose bisphosphatase. This enzyme remains active after removing the reductant, and deactivation is explainable by either a steep sensitivity of enzyme to pH changes [11,12], or by electron-transfer to oxidized thioredoxin [13]. Neither of these effects seems to be required to explain deactivation of chloroplastic NADP-malate dehydrogenase in the dark. The spontaneous and rapid deactivation of this enzyme upon removal of reductant, may be viewed as an efficient molecular device that prevents the use of malate from chloroplast and cytosol to generate chloroplastic NADPH in the dark.

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