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A thioredoxin-independent fully active NADP-malate dehydrogenase obtained by site-directed mutagenesis

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A triple cysteine mutant of sorghum leaf NADP-malate dehydrogenase has been constructed by site-directed mutagenesis, combining the previously obtained mutation of the two N-terminal cysteines with the mutation of the most internal of the two C-terminal cysteines. The construct, over-expressed in *E. coli*, yielded an always active, dithiol-insensitive enzyme. It can be concluded that the dithiol activation of the unmodified enzyme involves a maximum of two different disulfides per subunit, and that none of the mutated cysteines is implicated in catalysis.

Malate-dehydrogenase; Thioredoxin; Light activation; Disulfide and site-directed mutagenesis

1. INTRODUCTION

The NADP-dependent malate dehydrogenase (NADP-MDH) (EC 1.1.1.82) is present in the chloroplasts of mesophyll cells of all photosynthetic plants. It plays a major role in the CO_2 fixation pathway of C_4 species [1]. Unlike the always active, NAD-dependent forms, it is inactive in the dark and activated in the light. Previous studies established that the activation is correlated with a reductive cleavage of disulfide bonds on the inactive (oxidized) enzyme through a reversible dithiol/disulfide interchange with photosynthetically reduced thioredoxin m [2–4].

More detailed chemical modification studies on corn enzyme showed that a disulfide bridge located near the N-terminus of each subunit was reduced upon activation [5]. The occurrence of a unique N-terminal disulfide bridge was also reported for the pea chloroplast enzyme subunit [6]. However, chemical quantitation of thiols on the previously denatured, then chemically reduced, corn enzyme indicated the possible presence of 2 or even 3 disulfides per monomer [7], and cDNA sequencing revealed the existence of a total of 8 cysteines per subunit of either corn or sorghum maturated enzyme [8,9]. Compared to the sequences of the NAD-dependent enzymes, all the NADP-MDH's sequenced so far were shown to possess 2 extensions, one N-terminal, bearing the 2 previously identified regulatory cysteines, and the other C-terminal, bearing one cysteine, and bordered by another one which has no counterpart in the NAD-dependent forms. None of the 4 other cysteines which are present in the core part of the molecule is conserved in the NAD forms [8,9].

Recently, we confirmed the role of the N-terminal disulfide bridge in activation by performing site-directed mutagenesis on the full-length cDNA coding for sorghum leaf NADP-MDH [10], however, the replacement (separately or together) of the 2 N-terminal cysteines by serines did not suppress the requirement for reduced thioredoxin, but dramatically changed the time–course of activation: the activation process, which took more than 10 min to reach completion with the unmodified enzyme was almost instantaneous with the mutated protein. These results suggested that, while the reduction of the N-terminal disulfide bridge constituted the limiting step of the activation, the reduction of a second thioredoxin-reducible disulfide bridge was necessary to get an active enzyme. The involvement of two disulfides, with different reduction properties, was also proposed independently in two recent papers dealing, respectively, with maize leaf NADP-MDH [7] and pea leaf NADP-MDH [11], based on data obtained by a biochemical approach.

In the present study, we provide evidence that mutating one cysteine, located in the C-terminal part of the protein, in addition to the two cysteines of the N-terminal disulfide bridge, yields an always active, dithiol-insensitive enzyme.

2. MATERIALS AND METHODS

2.1. *Materials*

The *E. coli* strains used for high-yield plasmid production, dU-

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Abbreviations: DTT, dithiothreitol; NADP-MDH, NADP-dependent malate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; Tris, tri(hydroxymethyl) aminomethane; WT, wild-type.

substituted DNA template synthesis and mutated CM7 cDNA [9] expression were the same as in [10] and they were grown in similar conditions.

Spinach thioredoxin m was prepared as already described [12].

All enzymes for molecular biology as well as oligonucleotides for DNA sequencing and site-directed mutagenesis were purchased from Appligene, Boehringer-Mannheim, and Bethesda Research Laboratories. Radiolabels were from Amersham. Chromatographic supports were from Sigma and Grace-Amicon. Reagents for amino acid sequencing were supplied by Applied Biosystems Inc.

2.2. *Site-directed mutagenesis and construction of a cDNA coding for a mutant NADP-MDH with three substituted cysteines*

All methods for molecular biology are described in [13] The C405A mutation was introduced into the CM7 cDNA by the method described by Kunkel et al. [14] The following oligonucleotide: pGAGAAGAAAGCCGTTGCCCATC was used to replace the codon coding for Cys-405 (sequence numbering based on [9]) with a codon coding for an alanine (underlined). The *Bgl*II/*Xho*I fragment encompassing the mutagenesis site was transferred into the pETmdh expression system [10] using pUC9 as vehicle. Then, the *Apa*I cassette from the previously obtained C64/69S CM7 cDNA [10] was exchanged with the corresponding wild-type fragment. The DNA sequence was controlled at each cloning step.

2.3. *Production and purification of the modified NADP-MDH protein*

The protocols for expression of the mutated CM7 cDNA in the pET system [15], as well as for preparation of soluble protein extracts from *E coli* were the same as previously detailed [10] Proteins from the crude extract were fractionated by ammonium sulfate precipitation. The precipitate obtained between 35 and 60% of saturation was dissolved in 20 mM sodium phosphate (pH 7.2) buffer containing 1 mM EDTA (PE buffer) and dialyzed overnight against 50 vols of the same buffer. The fractionated extract was loaded onto a DEAE-Sephacel column (3 cm diameter, 22 cm long) equilibrated with PE buffer. After extensive washing of the column with the same buffer, elution was carried out with a linear gradient (2 × 250 ml) of 0–600 mM NaCl in PE buffer. Fractions enriched in NADP-MDH were pooled and directly loaded onto a Matrex red A column (1.5 cm diameter, 13 cm long). After washing with PE buffer, the enzyme was eluted from the affinity column with a linear gradient of 0–3 M NaCl (2 × 250 ml) in PE buffer The peak fractions were pooled and dialysed against 30 mM Tris-HCl, pH 7.4, 1 mM EDTA buffer. Then, the purified preparation was concentrated to 1 -1.5 mg protein/ml in an Amicon ultrafiltration cell equipped with a YM10 membrane.

2.4 *Characterization of the mutant NADP-MDH protein*

Enzyme activity assay, kinetic parameters determination and polyacrylamide gel electrophoresis were performed as in [10]. NADP-MDH was detected on gels by Western immunoblotting procedure already described in [16]. An Applied Biosystems model 476A sequencer with on line HPLC detection of phenylhydantoin amino acids was used for N-terminal amino acid sequence analysis of wild-type (WT) and mutant NADP-MDH.

3. RESULTS AND DISCUSSION

The triple cysteine mutant of sorghum leaf NADP-MDH was constructed by combining the previously constructed C64/69S mutant (numbering used in [9] and starting at the beginning of the transit peptide) lacking the N-terminal disulfide bridge [10] with the replacement of the most internal of the 2 C-terminal cysteines by an alanine (C405A). The decision to construct a combined mutation was reached because the single mutation C405A yielded a protein which still needed a

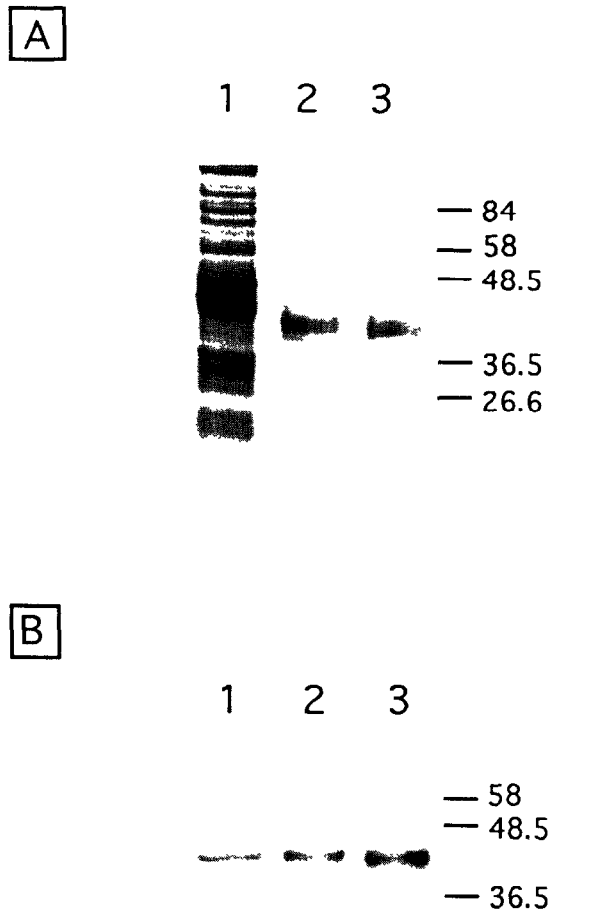

Fig. 1. (A) SDS-polyacrylamide gel electrophoresis (10%) stained by Coomassie brilliant blue. Lane 1, crude soluble extract of *E coli* producing the mutant NADP-MDH; 2, purified triple cysteine mutant NADP-MDH; 3, purified sorghum leaf NADP-MDH. (B) Immunoblotting of the same samples probed with anti-recombinant NADP-MDH IgG The positions of molecular mass standards are indicated in kDa.

preactivation with reduced thioredoxin to be active (data not shown). In the soluble crude extract of *E. coli* cells expressing the triple cysteine mutant cDNA, a strong NADP-MDH activity could be measured without any activating pretreatment, while all the NADP-MDHs expressed so far in *E. coli* needed a preactivation with reduced thioredoxin to be detectable [10]. The whole purification process could be followed by directly measuring the NADP-MDH activity. The chromatographic behaviour of the mutant protein was unchanged on ion-exchange chromatography on DEAE-Sephacel, but its elution from Matrex red A required higher NaCl concentrations (2.5 M instead of 1.3 M, data not shown) suggesting a more 'open' conformation towards the dye which mimicks the structure of the cofactor. After purification close to homogeneity, as judged by SDS-PAGE (Fig. 1A), the protein still exhibited high spontaneous activity. Its molecular mass was very similar to the one

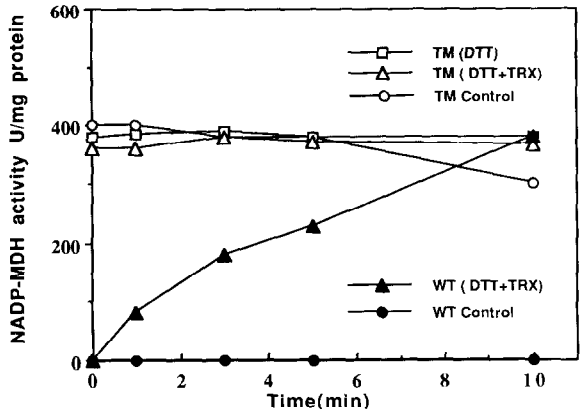

Fig 2. Effect of preincubation with dithiols on the activity of triple cysteine mutant (TM), compared to unmodified (WT) NADP-MDH. The enzymes were preincubated in 30 mM Tris buffer, pH 7.9, supplemented with 10 mM DTT (DTT) or 1 mM DTT + 15 μM thioredoxin (DTT + TRX) where indicated. Aliquots were withdrawn after the indicated times of incubation and assayed for activity.

of the protein purified from plant leaves, suggesting the processing of the recombinant preprotein in *E. coli*, as already observed for the recombinant WT enzyme [10].

This was confirmed by amino acid sequencing of the N-terminal part of the pure protein, which showed that the longest form which could be identified in the preparation started at Ser-41, as reported for the plant enzyme [9]. Like for the WT recombinant enzyme [10], forms shortened by several amino acids could also be detected, suggesting some N-terminal nonspecific proteolysis.

The mutated protein was recognized by antibodies raised against the WT recombinant protein (Fig. 1B). In crude extracts, a faint higher molecular weight band could also be detected. Taking into account the predicted size of the transit peptide [9], it could correspond to the unprocessed form of the protein. It has never been visualized in plants, nor in our previous, less efficient expression system [17], however, it was always present in crude extracts when the improved pETmdh expression system was used [10], and lost during purification.

Table I

Effect of preincubation with NADP on the activity of the triple cysteine mutant NADP-MDH

Time of preincubation (min)	NADP-MDH activity (U/mg protein)	
	No NADP	400 μM NADP
0	400	400
1	400	400
5	380	380

The enzyme was preincubated with or without NADP, in a 30 mM Tris buffer, pH 7.9, 10 mM DTT and 15 μM thioredoxin. Aliquots were removed periodically and assayed for activity. The same results were obtained when the reductant was omitted from the medium

The specific activity of the purified mutant protein ranged from 400 to 500 U/mg, similar to the values reported for the WT protein. The K_m for the substrates were also in the range of the standard WT values [10] (data not shown). Clearly, the mutation did not alter the catalytic properties of the enzyme, but simply locked it in a fully activated conformation. As a matter of fact, treatments known to affect, either positively or negatively, the activation of the enzyme had no effect on the mutant. Thus, the activity could not be increased by preincubation with either DTT or DTT-reduced thioredoxin (Fig. 2). On the other hand, preincubation with NADP, a well-known inhibitor of activation [18] did not decrease the activity (Table I). This confirms the previous assumptions that NADP prevented the enzyme attaining an active conformation but did not interfere with catalysis.

Taken together with our previous findings [10], these results provide evidence that the activation of NADP-MDH involves the reduction of no more than 2 disulfides per subunit. Besides the previously identified N-terminal disulfide, the reduction of which is rate-limiting for activation, an additional disulfide, involving Cys-405, must certainly be reduced. The full demonstration of this will be achieved after identification of the partner cysteine of Cys-405. Further mutagenesis experiments are in progress for this purpose. Considering the better binding of the triple cysteine mutant to triazine dye columns, it can be speculated that the opening of the second disulfide facilitates the access of NADPH. From present evidence, we can conclude that Cys-405 has no catalytic function, as its replacement by an alanine yields a fully active enzyme.

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REFERENCES

[1] Hatch, M D. and Slack, C R. (1970) Annu. Rev. Plant Physiol. 21, 141–159.
[2] Jacquot, J.P., Gadal, P., Nishizawa, A.N., Yee, B.C , Crawford, N.A. and Buchanan, B.B. (1984) Arch. Biochem. Biophys. 228, 170–178.
[3] Buchanan, B B. (1980) Annu. Rev. Plant Physiol. 31, 341–374.
[4] Droux, M., Miginiac-Maslow, M , Jacquot, J.P., Gadal, P., Crawford, N.A., Kosower, N.S. and Buchanan, B.B. (1987) Arch. Biochem. Biophys. 256, 372–380.
[5] Decottignies, P., Schmitter, J.M., Miginiac-Maslow, M., Le Marechal, P., Jacquot, J P. and Gadal, P. (1988) J. Biol. Chem. 263, 11780–11785.
[6] Scheibe, R., Kampfenkel, K., Wessels, R. and Tripier, D. (1991) Biochim. Biophys. Acta 1076, 1–8.
[7] Hatch, M D. and Agostino, A. (1992) Plant Physiol. 100, 360–366.
[8] Metzler, M.C., Rothermel, B.A. and Nelson, T. (1989) Plant Mol. Biol. 12, 713–722
[9] Crétin, C., Luchetta, P., Joly, C., Decottignies, P., Lepiniec, L., Gadal, P., Sallantin, M., Huet, J.C and Pernollet, J.C. (1990) Eur J. Biochem. 192, 299–303.

[10] Issakidis, E., Miginiac-Maslow, M., Decottignies, P., Jacquot, J.P., Crétin, C. and Gadal, P. (1992) J. Biol. Chem. 267, 21577–21583.
[11] Kampfenkel, K. (1992) Biochim. Biophys. Acta 1156, 71–77.
[12] Schürmann, P. and Jacquot, J.P (1979) Biochim. Biophys. Acta 569, 309–312.
[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY.
[14] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[15] Studier, F.W., Rosenberg, A.H., Dunn, J.J and Dubendorff, J.W. (1990) Methods Enzymol. 185, 60–89.
[16] Crétin, C., Luchetta, P., Miginiac-Maslow, M., Decottignies, P., Jacquot, J.P., Vidal, J. and Gadal, P. (1988) Eur. J. Biochem. 174, 497–501.
[17] Jacquot, J.P., Keryer, E., Issakidis, E., Decottignies, P., Miginiac-Maslow, M., Schmitter, J.M. and Crétin, C. (1991) Eur. J. Biochem. 199, 47–51.
[18] Scheibe, R. and Jacquot, J.P. (1983) Planta 157, 548–553.