An active-site cysteine of sorghum leaf NADP-malate dehydrogenase studied by site-directed mutagenesis**

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Abstract The chloroplast NADP-malate dehydrogenase is activated through the reduction of two different disulfides per subunit. The activated enzyme, as well as a permanently active mutant where all four regulatory cysteines were replaced are still sensitive to thiol reagents. This observation suggested the presence of an additional important cysteine at the active site. In an attempt to identify that cysteine, site-directed mutagenesis was performed on the cDNA encoding sorghum leaf NADP-malate dehydrogenase. The replacement of Cys-175 by an alanine yielded an enzyme whose sensitivity to thiol reagents was markedly decreased whereas its catalytic activity was enhanced. This finding suggests that Cys-175 has no catalytic function but is located close to the active site.

Key words: Malate dehydrogenase; Thioredoxin; Light-activation; Disulfide mutagenesis; Site-directed mutagenesis

1. Introduction

Among all the malate dehydrogenases studied so far, the chloroplastic isoforms exhibit unique properties: they use NADPH instead of NADH as a cofactor and are thiol-regulated. They are totally inactive in the oxidized form, and activated upon reduction by reduced thioredoxin. The activation of sorghum leaf NADP-malate dehydrogenase (NADP-MDH) (EC 1.1.1.82) has been demonstrated to result from the reduction of two disulfide bridges per subunit, one located at the N-terminus [1,2] and the other at the C-terminus [3]. The regulatory cysteines have no catalytic function, as their replacement by either serines or alanines does not impair the activity but yields a deregulated, permanently active enzyme [3]. Furthermore, the presence of an essential histidine at the active site of the enzyme has been demonstrated [4]. This histidine is strictly conserved among all the malate dehydrogenases sequenced so far [5] and is considered to be involved in catalysis, its replacement by an alanine yielding a totally inactive protein. Nevertheless, the catalytic activity of the activated wild-type (WT) enzyme is known to be sensitive to thiol reagents [2,6,7]. Preliminary experiments showed that the permanently active mutant NADP-MDH where all four regulatory cysteines were substituted could also be inhibited by the thiol reagent iodoacetamide [3]. In addition, molecular modeling indicated the presence of a cysteine within the active site of the enzyme, in close vicinity to the coenzyme nicotinamide ring [3]. This cysteine was numbered 215 following the numbering in [8], including the 40 amino acids of the transit peptide of this nuclear-encoded chloroplastic enzyme, which corresponds to position 175 in the mature protein [4]. In the active sites of NAD-malate dehydrogenases (which are permanently active), there is an alanine at this position instead, despite the high degree of similarity of both types of active sites.

In the present study, we further examined the possible role of a cysteine at the active site of NADP-malate dehydrogenase, by investigating whether the presence of substrates protected the permanently active mutant against inactivation by thiol reagents. We also replaced, in the wild-type enzyme, the putative active-site Cys-175 with an alanine, by site-directed mutagenesis, and studied the properties of the mutant protein.

2. Materials and methods

2.1. Materials

The *E. coli* strains used for high yield plasmid production, dU-substituted DNA template synthesis and mutated sorghum CM7 cDNA [8] expression were the same as in [2] and were grown under similar conditions. Recombinant thioredoxin from *E. coli* was prepared as described in [9].

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, Inc. Radiolabels were from Amersham Corp. 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 the substitution C175A

All methods for molecular biology are described in [10]. The C175A mutation was introduced into the CM7 cDNA (full-length cDNA coding for sorghum leaf chloroplastic NADP-malate dehydrogenase, including the sequence coding for the transit peptide) by the method described by Kunkel et al. [11]. The following oligonucleotide: pGGAAATCCCGCTAACACTA was used to replace the codon coding for Cys-175 (sequence numbering as in [4]) with a codon coding for an alanine (underlined). After verification of the mutation by sequencing, the *NcoI/BglII* fragment encompassing the mutagenesis site was exchanged with the corresponding wild type fragment in the pETmdh expression vector [2]. The cDNA sequence was controlled at each cloning step.

The construction of the permanently active 4-cysteine mutant NADP-MDH has been described previously [3]. In this mutant, the two most N-terminal Cys were replaced by Ser and the two most C-terminal Cys were replaced by Ala.

2.3. Production and purification of the WT and mutated NADP-MDH proteins

The expression of the WT or mutated cDNAs in the pET system

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**This paper is dedicated to the memory of Markku Saarinen.

Abbreviations: DTT, dithiothreitol; NADP-MDH, NADP-dependent malate dehydrogenase; OAA, oxaloacetate; PMSF, phenylmethylsulfonyl fluoride; WT, wild-type.

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[12], as well as preparation of soluble protein extracts from *E. coli* were performed as previously described [2]. Crude extract proteins 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 100 μM PMSF 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 WT or C175A mutant enzyme was eluted from the affinity column with a linear gradient of 0–3 M NaCl (250/250 ml) in PE buffer. For the permanently active 4-Cys mutant, the gradient was extended to 4 M NaCl. The peak fractions were pooled and dialysed against cold 30 mM Tris-HCl, pH 7.4, 1 mM EDTA buffer. Then, the purified preparations were concentrated in an Amicon ultrafiltration cell equipped with a YM10 membrane to 1–1.5 mg protein/ml. When a perfectly homogeneous protein was required, an additional purification step was carried out by hydrophobic interaction on an HPLC phenyl 5PW column.

2.4. Characterization of the mutant NADP-MDH protein

Enzyme activity assays, kinetic parameter determinations and polyacrylamide gel electrophoresis were performed as in [2,13]. An Applied Biosystems model 476A sequencer with on line HPLC detection of phenylhydantoin aminoacids was used for N-terminal amino acid sequence analysis of WT and mutant NADP-MDH.

3. Results and discussion

Our previous mutagenesis experiments [3] demonstrated that the cysteines implicated in the regulatory disulfides were not involved in catalysis. Nevertheless, the activity of the permanently active 4-Cys mutant enzyme was inhibited by the thiol reagent iodoacetamide (Fig. 1). If the observed effect were due to the derivatization of an active-site cysteine, protection against inhibition should be observed in the presence of the substrates of the reaction. This was indeed the case, at least for the coenzyme (either NADP, as shown in Fig. 1, or NADPH, data not shown) the addition of which provided full protection of the activity. Furthermore, the mutant enzyme was obviously stabilized under these conditions, as the presence of the cofactor prevented the slow inactivation which usually occurs with this rather unstable mutant enzyme upon incubation at ambient temperature in a diluted form (Fig. 1). In contrast, oxaloacetate did not provide any significant protection (data not shown).

A definite proof of the occurrence of a thiol-reagent sensitive cysteine at the active site of the enzyme can be provided by site-directed mutagenesis. This was done by replacing Cys-175 of the WT enzyme by an Ala, the most commonly found amino acid at this place in the NAD-dependent forms. The mutant enzyme was overproduced in *E. coli* and purified close

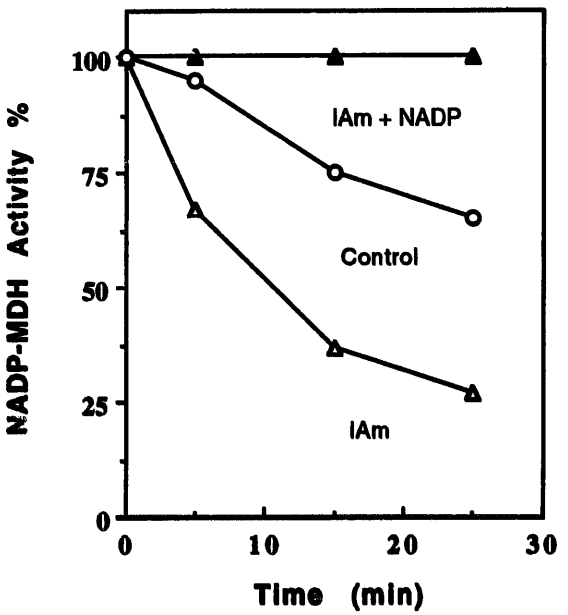

Fig. 1. Inactivation of the permanently active 4-Cys mutant NADP-malate dehydrogenase upon incubation with iodoacetamide (3 mM). Protection by preincubation with NADP (350 μM). 100% activity corresponds to a specific activity of 380 U/mg protein, assayed under standard conditions (750 μM oxaloacetate and 140 μM NADPH in 100 mM Tris-HCl buffer, pH 8). IAm = iodoacetamide.

to homogeneity. Then its physical and biochemical properties were studied and compared to the properties of the WT protein. The molecular mass of the mutant enzyme was identical to the one of the WT protein (42 kDa per subunit, when determined on SDS-PAGE). The determination of the N-terminal amino acid sequence showed that the amino-terminus of the purified protein started mainly at Ser-1 of the mature protein, i.e. that the transit peptide had been cleaved by the bacteria, as for all the other recombinant NADP-MDHs we studied so far. Like the WT protein, the mutant MDH behaved as a dimer on size-exclusion HPLC (data not shown). It was totally inactive in the oxidized form and required thioredoxin (reduced either by DTT, or by light in a reconstituted light-activation system) for activation, with half-saturation values of ca. 8 μM [14] as for the WT protein. The time course of activation was very similar to the one of the WT protein, the maximal activity being reached after ca. 10 min. The activation was accompanied by the appearance of 4 free cysteines per subunit (data not shown), thus the reduction of the regulatory disulfides was not disturbed by the mutation. As already reported for the WT protein [15], the activation was inhibited in the presence of NADP, the oxidized form of the cofactor (data not shown). Once activated,

Table 1
Kinetic parameters of WT and C175A mutant NADP-malate dehydrogenase

Enzyme	K_m (μM)		k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	
	OAA	NADPH		OAA	NADPH
WT	37 ± 5	40 ± 8	630 ± 70	18.6	17
C175A	32 ± 3	150 ± 5	3145 ± 360	98	21

The overall catalytic efficiencies [$k_{cat}/(K_m$ OAA)×(K_m NADPH)] of the WT and mutant MDHs calculated from these data are 4.25×10^{-11} and 6.55×10^{-11} $M^{-2} \cdot s^{-1}$, respectively. The enzymes were activated by 10 min preincubation with 10 mM DTT and 10 μM thioredoxin from *E. coli* in 100 mM Tris-HCl buffer, pH 8 before activity measurements. K_m for oxaloacetate was measured by varying oxaloacetate concentrations at a fixed (140 or 220 μM) NADPH concentration. K_m for NADPH was measured at 750 μM oxaloacetate concentration.

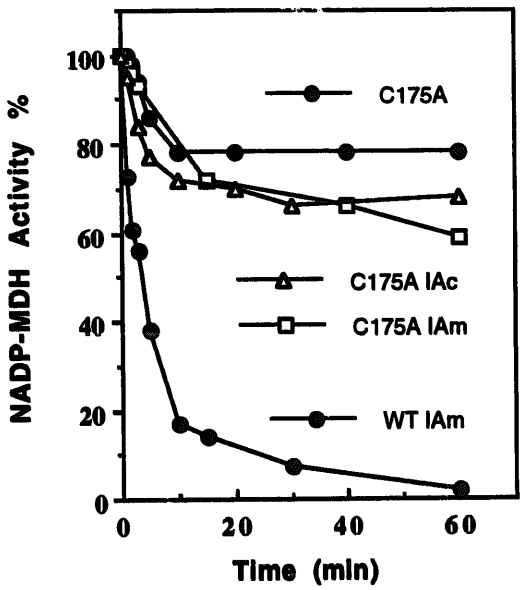

Fig. 2. Compared sensitivity of the C175A mutant and WT NADP-malate dehydrogenase to thiol reagents. Each enzyme (3–5 μg) was first activated by 10 min preincubation with 10 mM DTT and 10 μM thioredoxin from *E. coli* in 100 mM Tris-HCl buffer, pH 8 in a total volume of 100 μl. Then the thiol reagents were added in a 3 mM excess over the thiol content of dithiothreitol. The enzyme activities were assayed on aliquots under standard assay conditions, but with a 220 μM NADPH concentration. The untreated activated WT enzyme lost about 20% activity when kept at ambient temperature for 1 h. IAm = iodoacetamide. IAc = iodoacetate.

the enzyme exhibited a high specific activity: ca. 1000 U/mg for the mutant protein vs. 450 U/mg for the WT protein, when measured under the standard assay conditions (those used for the WT protein). When its kinetic parameters were determined, it appeared that whereas its K_m OAA was very close to that of the WT enzyme, its K_m NADPH was roughly 4 times higher (Table 1). Thus, from the V_{max} calculated from double-reciprocal plots a theoretical specific activity of 2300 U/mg and a k_{cat} of 3145 s^{-1} (vs. 630 U/mg for the WT enzyme) could be deduced. Clearly, the replacement of the cysteine did not impair, but rather increased the catalytic activity. However, it seemed to decrease the efficiency of coenzyme use. This observation suggests a possible role of the cysteine in coenzyme binding, in accordance with its predicted position, close to the nicotinamide ring of the cofactor [3,16].

To check whether the afore-mentioned sensitivity to thiol reagents of the activated NADP-MDH is due to this cysteine, the sensitivities of the activated WT enzyme and C175A mutant to iodoacetamide were compared. The results show (Fig. 2) that the sensitivity of the C175A mutant MDH to the reagent is dramatically decreased. In fact, its discrete loss in activity seems to be due more to partial inactivation upon storage of the activated enzyme at ambient temperature than to a specific effect, as the control sample where the reagent was replaced by buffer also presents a gradual loss in activity. Furthermore, iodoacetate (the negatively charged equivalent of iodoacetamide) which was widely used as a thiol-derivatizing agent for the WT protein [1] was equally unefficient. It has already been reported that the WT enzyme is more unstable when activated [7]. When iodoacetamide-treated activated C175A mutant was dialyzed to eliminate excess reagent, it remained active for several hours with little (ca. 15%) decrease in activity (data not shown), thus confirming that the derivatization of the regulatory cysteines is not harmful for activity.

In conclusion, all these results indicate that Cys-175 is the active site cysteine of NADP-MDH which is accessible to thiol reagents when the enzyme is activated (active site in an open conformation) and which is the main target of these reagents. However, the activity of the enzyme is not impaired upon mutation: on the contrary, its specific activity is increased several-fold. Thus, it is clear that this cysteine does not directly participate in catalysis. As a consequence, the existence of a catalytic triad (His,Asp,Cys) proposed by Jackson et al. [16] can be ruled out. The inhibitory effect of iodoacetamide on the activated WT or the permanently active four-Cys mutant seems to be rather due to the steric hindrance linked to the alkylation of this cysteine. A similar situation has been reported for dogfish lactate dehydrogenase [17] where the derivatization of a cysteine located at the active site results in an inhibition of the catalytic activity: the modification of this Cys prevents the conformational change necessary to the formation of the catalytic complex. In contrast, in aldehyde dehydrogenases, an active site Cys residue acts as a nucleophile and its mutation to an Ala yields a totally inactive enzyme [18]. In the case of NADP-malate dehydrogenase, the increase in K_m for NADPH upon changing the active-site Cys to an alanine suggests that this cysteine can be involved in coenzyme binding, which could explain its strict conservation among all the NADP-dependent forms. The lower K_m for NADPH in the WT enzyme is accompanied by a decreased k_{cat}: this feature seems to constitute a compensatory change, as the overall catalytic efficiencies of the WT and mutated enzymes (Table 1) are rather similar.

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References

[1] Decottignies, P., Schmitter, J.M., Miginiac-Maslow, M., Le Marechal, P., Jacquot, J.P. and Gadal, P. (1988) J. Biol. Chem. 263, 11780–11785.
[2] Issakidis, E., Miginiac-Maslow, M., Decottignies, P., Jacquot, J.P., Crétin, C. and Gadal, P. (1992) J. Biol. Chem. 267, 21577–21583.
[3] Issakidis, E., Saarinen, M., Decottignies, P., Jacquot, J.-P., Crétin, C., Gadal, P. and Miginiac-Maslow, M. (1994) J. Biol. Chem., 269, 3511–3517.
[4] Lemaire, M., Schmitter, J.-M., Issakidis, E., Miginiac-Maslow, M., Gadal, P. and Decottignies, P. (1994) J. Biol. Chem. 269, 27291–27296.
[5] Goward, C.R. and Nicholls, D.J.(1994) Protein Sci. 3, 1883–1888.
[6] Ashton, A.R. and Hatch, M.D. (1983) Arch. Biochem. Biophys. 227, 406–415.
[7] Scheibe, R., Geissler, A. and Rother, T. (1993) Arch. Biochem. Biophys. 300, 635–640.
[8] Cretin, 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.
[9] De Lamotte-Guéry, F., Miginiac-Maslow, M., Decottignies, P., Stein, M., Minard, P. and Jacquot, J.-P. (1991) Eur. J. Biochem. 196, 287–294.
[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[11] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.

[12] Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Methods Enzymol. 185, 60–89.
[13] Jacquot, J.P., Keryer, E., Issakidis, E., Decottignies, P., Miginiac-Maslow, M., Schmitter, J.M. and Cretin C. (1991) Eur. J. Biochem. 199, 47–51.
[14] Issakidis, E., Lemaire, M., Decottignies, P., Ruelland, R., Jacquot, J.-P. and Miginiac-Maslow, M. (1995) in: Photosynthesis: From Light to Biosphere, vol. V (Mathis, P. ed.) pp. 83–86, Kluwer, Dordrecht.
[15] Scheibe, R. and Jacquot, J P. (1983) Planta 157, 548–553.
[16] Jackson, R.M., Sessions, R.B. and Holbrook, J.J. (1992) J. Computer-Aided Mol. Design 6, 1–18.
[17] Holbrook, J.J., Liljas, A., Steindel, S.J. and Rossmann, M.G. (1975) in: The Enzymes, vol. XI A (Boyer, P. ed.) 3rd Edn, pp. 191–292, Academic Press, New York.
[18] Farres J., Wang T.T.Y., Cunningham S.J. and Weiner H. (1995) Biochemistry 34, 2592–2598.