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Direct evidence for the different roles of the N- and C-terminal regulatory disulfides of sorghum leaf NADP-malate dehydrogenase in its activation by reduced thioredoxin

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Abstract Plant NADP-dependent malate dehydrogenase is activated through thiol/disulfide interchange with reduced thioredoxin. Previous studies showed that this process involves the reduction of two different disulfides per subunit: one N-terminal, the other C-terminal. Substitution of regulatory cysteines at each end by site-directed mutagenesis and comparison of activation kinetics of the mutants led us to propose a model for the activation mechanism where the C-terminal end shielded the access to the catalytic residues, whereas the N-terminal end was involved in the slow conformational change of the active site. In the present study, we took advantage of the previous identification of the catalytic histidine residue which can be specifically derivatized by diethyl pyrocarbonate to test the accessibility of the active site. The results clearly show that in the mutants where the C-terminal bridge is open the active site histidine is freely accessible to the reagent, whereas in the mutants where the N-terminal bridge is open, the active site cannot be reached without activation, thus demonstrating the validity of the model.

Key words: NADP-malate dehydrogenase; Light activation; Site-directed mutagenesis; Thiol; Thioredoxin

1. Introduction

The major functional peculiarity of the chloroplastic NADP-dependent malate dehydrogenase (NADP-MDH: EC 1.1.1.82) resides in its activation properties. Unlike its NAD-dependent ubiquitous counterparts, it is totally inactive when oxidized, and fully active when reduced. This redox dependent process is mediated by thiol-disulfide interchange with the very reactive disulfide bridge of thioredoxin. It constitutes the physiological means of regulation of the enzyme activity in vivo by switching it on (in the light) or off (in the dark) in accordance with the availability of the reducing power [1]. In a recent work [2–4], we showed that two different disulfides have to be reduced per subunit of this homodimeric enzyme during the activation process. Both are located in sequence extensions specific for the redox-regulated forms: one at the N-terminus, the other at the C-terminus [5]. By using site-directed mutagenesis techniques to substitute the cysteines at each end, we could obtain mutated enzymes bearing only one of the disulfides and study their activation kinetics. In the absence of the N-terminal disulfide, the reduction of the C-terminal bridge led to instantaneous activation of the enzyme. In contrast, when the C-terminal bridge was suppressed, the activation kinetics was slow: full activation took about 10–15 min, as for the unmodified protein. This observation prompted us to propose different roles for the N- and C-terminal disulfides in activation. The reduction of the C-terminal disulfide was proposed to uncover access to the active site, whereas the reduction of the N-terminal disulfide would trigger a conformational change shaping the active site in a fully active conformation. Further studies [6,7] identified His-229 and Asp-201 as the catalytically active residues, forming a proton relay system, as in the NAD-dependent dehydrogenases [8]. These residues are strictly conserved among all the malate dehydrogenases [9]. His-229 was shown to be specifically derivatized with diethylpyrocarbonate (DEPC), leading to the complete loss of catalytic activity [6]. The derivatization required preactivation of the enzyme, suggesting that in the inactivated protein, the catalytic site was screened from the solvent. Thus, DEPC treatment before or after activation seemed to be a good probe to check the accessibility of the active site. In the present study, we took advantage of this observation to explore further the different roles of the N- and C-terminal disulfides in the activation process of sorghum leaf recombinant NADP-MDH.

2. Materials and methods

2.1. Production of WT and mutant proteins

The WT and mutant recombinant proteins were obtained as described [2,4]. The site-directed mutagenesis technique of Kunkel [10] was used to introduce mutations in the WT [5] cDNA. The different cDNAs were cloned in the pET vector [11] and expressed in *E. coli* BL21 (DE3) strain.

Among all the mutants created previously, three were retained for the present study: the N-terminal double cysteine mutant (NDM, corresponding to the mutation C24S/C29S), the C-terminal double cysteine mutant (CDM, corresponding to the mutation C365A/C377A) and the quadruple cysteine mutant (QM) combining the mutations at both ends. It must be noted that the cysteine numbering differs from that used previously [2–4] by 40 residues. This difference corresponds to the residues for which it has been firmly established now that they belong to the transit peptide which is cleaved in the mature protein.

2.2. Purification of the recombinant proteins

The expressed proteins were purified to homogeneity by previously

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Abbreviations: CDM, double mutant NADP-malate dehydrogenase, mutated on both C-terminal cysteines; NDM, double mutant NADP-malate dehydrogenase mutated on both N-terminal cysteines; DEPC, diethylpyrocarbonate; DTT, dithiothreitol; NADP-MDH, NADP-malate dehydrogenase; QM, quadruple mutant NADP-MDH, mutated on the four regulatory cysteines

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described procedures [2–4] combining ammonium sulfate fractionation, ion-exchange chromatography on DEAE-Sephacel, affinity chromatography on Matrex redA and hydrophobic interaction on phenyl-Sepharose HPLC. The N-terminus of each mutant protein was verified by amino-terminal sequencing: in all cases, the transit peptide was removed by the bacteria, but the regulatory amino-terminal extension was retained.

Recombinant *E. coli* thioredoxin was purified as described previously [12].

2.3. Standard assay for NADP-MDH activity

The enzymes were preactivated by incubation in the presence of 10 mM DTT and 20 μM thioredoxin from *E. coli* in pH 8, 100 mM Tris-HCl buffer. The activity was measured on aliquots (corresponding to approx. 0.4 μg MDH) added to a spectrophotometer cuvette containing 1 ml of reaction medium (140 μM NADPH, 760 μM oxaloacetate in 100 mM Tris-HCl buffer, pH 8). In the case of the permanently active quadruple cysteine mutant, the reaction was started by adding the enzyme directly to the cuvette, without preincubation. The same procedure was followed when the spontaneous activity of the CDM was measured, but in this case, the oxaloacetate concentration was raised to 5 mM. The activity was estimated by the decrease in absorbance at 340 nm.

2.4. DEPC treatment

The sensitivity of the WT and mutant NADP-MDH to DEPC was tested on either unactivated or previously activated enzymes. In the first case, the enzymes were preincubated with DEPC (freshly diluted in ethanol) for 5 min in 20 mM sodium phosphate buffer, pH 7.2. Then, the activation medium (10 mM DTT, 20 μM thioredoxin in 100 mM Tris-HCl buffer, pH 8) was added, causing 5-fold dilution of the reagent. The activation of the enzyme was then measured as a function of time, on aliquots. The control sample was treated with an equivalent volume of ethanol (10% final concentration). In the second case, the enzymes were preactivated by incubation with DTT and thioredoxin in 20 mM sodium phosphate buffer, pH 7.2. When full activation was reached, DEPC (or ethanol for the control) was added and MDH activity measured as a function of time, on aliquots.

2.5. Preparation of DEPC-derivatized NADP-MDH: substrate protection

To investigate the stability of the derivatization as well as the protective effect of NADPH, the unactivated CDM enzyme (300 μg in 500 μl of 20 mM phosphate buffer, pH 7.2) was pretreated with 1 mM DEPC, in the presence or absence of 1 mM NADPH. The unreacted reagent and cofactor were eliminated by centrifugation and extensive washing with 20 mM phosphate buffer pH 7.2 on Amicon Centricon 30 concentrators (30 kDa cut-off). The dialyzed enzyme was subjected to activation with reduced thioredoxin, at pH 8, and its activity was measured on 0.4 μg aliquots.

3. Results and discussion

The previously created WT or mutant NADP-MDHs [2–4] were used for this investigation. The cDNAs were overexpressed in *E. coli* using the very efficient pET expression system [11]. Since BL21(DE3) is not a RecA$^-$ strain, the host bacteria were freshly transformed every time a new protein preparation was needed. The most productive clones (based on activity measurements in crude extracts) were retained for large scale (8 l) cultures and protein purification to homogeneity by previously described procedures [2,4]. The mutated proteins were either double-cysteine mutations of the N-terminal bridge (NDM) or the C-terminal bridge (CDM), or a quadruple mutation (QM) where both disulfides were substituted (see methods for cysteine numbering). As shown previously [4], the last mutant is a permanently active, thioredoxin-independent enzyme.

The inhibitory effect of DEPC was first examined on fully activated proteins. DEPC being unstable under alkaline conditions, the preactivation was performed in phosphate buffer, pH 7.2, instead of pH 8 Tris-HCl buffer. When full activation was reached, DEPC was added, and the activity of the enzymes was measured on aliquots. Fig. 1 shows that all the enzymes were inhibited by DEPC, although with somewhat varying sensitivities. The N-terminal double mutant (NDM) behaved much the same way as the WT protein (50% inhibition for 1 mM DEPC; 75% inhibition for 2 mM DEPC; full inhibition (not shown) required 4 mM concentration). The C-terminal double mutant (CDM) was slightly more sensitive (75% inhibition for 1 mM DEPC). The most efficient inhibition (over 95% with 1 mM DEPC) was achieved for the permanently active quadruple cysteine mutant. However, it should be borne in mind that it is permanently active and did not require the addition of DTT and thioredoxin. Consequently, the access of the reagent to the active site of this mutant could be facilitated. Thus, besides somewhat different sensitivities to the reagent, all the activated, or permanently active, enzymes were inhibited by DEPC, like their NAD-dependent counterparts [13,14]. The results were quite different when DEPC was applied before activation (Fig. 2). The WT and NDM enzymes which have no activity without activation remained inactive after a 5 min DEPC treatment, but could be fully activated by the further addition of DTT and thioredoxin in pH 8 Tris buffer. This addition diluted the DEPC from 1 mM to 0.2 mM. Moreover, the reagent is unstable at pH 8. This explains why no inhibition was observed after activation, and demonstrates that the active site of the unactivated NDM could not be reached by the reagent. In contrast, the faint spontaneous activity of the unactivated CDM protein which is observed at high oxaloacetate concentration [4] was almost fully inhibited by DEPC. The inhibition was not relieved by the further addition of the activation medium: the pretreated

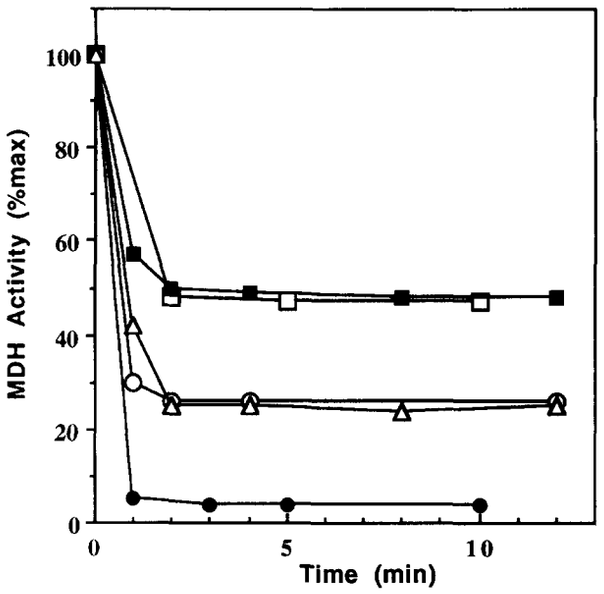

Fig. 1. Inhibition of fully activated (or permanently active) NADP-MDHs by DEPC. All the enzymes (except the permanently active QM) were first activated in the presence of 10 mM DTT and 20 μM thioredoxin in 20 mM phosphate buffer pH 7.2. Then 1 mM DEPC was added and the activity measured on aliquots. The activity is expressed as % of the activity of the fully activated enzymes: (□) WT enzyme; (●) QM; (■) NDM; (○) CDM; (△) NDM, DEPC 2 mM. The control samples (not shown for clarity) supplemented with an equivalent amount of ethanol did not show more than a 5% decrease in activity within 10 min.

enzyme could no longer be activated. Obviously, in this case, the catalytic histidine residue has been stably derivatized before activation. Thus, in the unactivated CDM, the active site seems to be freely accessible to the reagent.

Previous analytical work [6] based on tryptic digestion of the DEPC-derivatized protein and mass spectrometry analyses of the tryptic peptides showed unambiguously that in the activated WT protein only the active-site His was derivatized by DEPC. A good indication that the primary target of the reagent is indeed an active-site residue is provided by substrate protection experiments. In the case of WT NADP-MDH, a good protection against inhibition was obtained by preincubation with NADPH [6]. All the mutant enzymes tested in the present work could be protected against DEPC inhibition by 1 mM NADPH, as well as the WT protein (data not shown). In the case of the CDM and the QM whose activation (or permanent activity) is not inhibited by NADP, the oxidized cofactor was also efficient in protection (data not shown). We took advantage of the substrate protection effect to confirm the accessibility of the active site to DEPC in the unactivated CDM and to assess the stability of the derivatization (Fig. 3). The CDM enzyme was preincubated with DEPC, with or without NADPH. After 5 min, the samples were diluted with phosphate buffer and extensively dialyzed against the same buffer to eliminate the DEPC and NADPH. The dialyzed proteins were then supplemented with the activation medium and their activity measured as a function of time. The sample previously incubated with NADPH could be fully activated. The control sample remained inactive, showing that the DEPC treatment led to a covalent modification of the catalytic histidine residue.

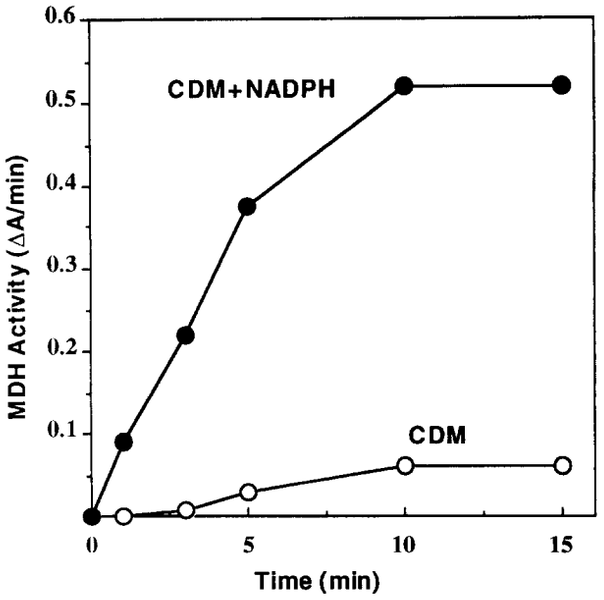

Fig. 3. Activation kinetics of the DEPC-pretreated, then dialyzed CDM malate dehydrogenase. Protective effect of NADPH. The CDM was preincubated with 1 mM DEPC for 5 min in the presence or in the absence of 1 mM NADPH. In the presence of NADPH, its spontaneous activity, observed at high oxaloacetate concentration (see Fig. 2), was retained, whereas it disappeared in the absence of the cofactor. The pretreated proteins were dialyzed extensively. Then the activation medium was added and the activity measured in standard conditions (i.e., low oxaloacetate: this explains why the zero time activity in the presence of NADPH is very low). The activity is expressed as the decrease in absorbance at 340 nm.

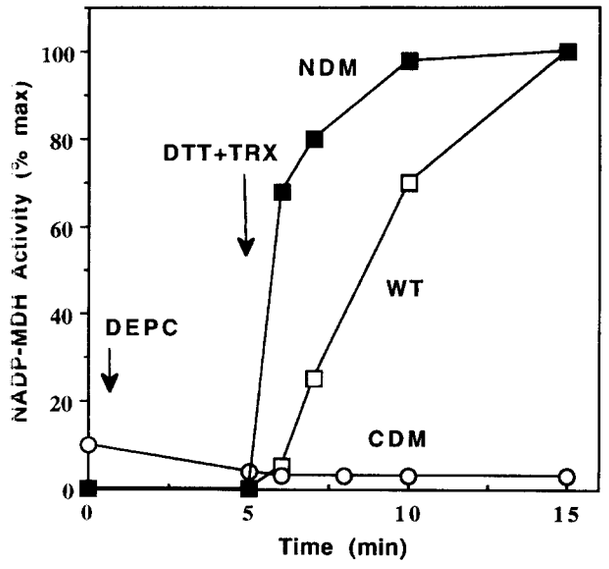

Fig. 2. Effect of previous treatment with DEPC on further activation of WT and mutant NADP-MDHs. The proteins were preincubated for 5 min with 1 mM DEPC in 20 mM phosphate buffer pH 7.2. Then the activation medium (10 mM DTT+20 μM thioredoxin in 100 mM Tris-HCl buffer pH 8) was added, diluting the DEPC 5-fold. The activity is expressed as % of the activity of the fully activated proteins. Before the addition of DEPC, the spontaneous activity of the different proteins was measured. Only the CDM exhibited a faint spontaneous activity before activation: it reached 8% of the activity of the fully activated protein when oxaloacetate concentration was raised to 5 mM. All the other activity measurements were performed at standard (760 μM) oxaloacetate concentration, as the activated enzymes are inhibited by excess oxaloacetate.

In conclusion, the present results constitute the first direct evidence for the different spatial roles of the N- and C-terminal disulfides in the activation of NADP-MDH. They clearly demonstrate that the opening of the N-terminal disulfide bridge does not give access to the active site. In contrast, when the C-terminal disulfide is open, the active site histidine is fully accessible in the absence of substrates and can be specifically and stably derivatized even when the enzyme has not been activated.

These results validate the previously proposed activation model [4] where the reduction of the C-terminal bridge opens the access to the active site whereas the reduction of the N-terminal bridge yields a slow conformational change giving the active site a fully active conformation. The precise nature of this conformational change is still to be defined. The cysteines of the thioredoxin-dependent regulatory disulfides are not involved in catalysis [4] and an internal cysteine located at the active site can be substituted without major consequences for activity [15]. The other well-documented thioredoxin-dependent activation mechanism of a chloroplastic enzyme concerns phosphoribulokinase. In this case, only one disulfide bridge is reduced upon activation and both regulatory cysteines are located at the active site [16]. For the other thiol-regulated chloroplastic enzymes, the sequence of events leading to activation has not been clearly identified yet. Thus, in the present state of the art, the very sophisticated activation mechanism of NADP-malate dehydrogenase is rather unique among the thioredoxin activated chloroplastic enzymes.

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