Sites of interaction of thioredoxin with sorghum NADP-malate dehydrogenase

Aymeric Goyer[a], Paulette Decottignies[b], Emmanuelle Issakidis-Bourguet[a], Myroslawa Miginiac-Maslow[a,*]

[a] *Institut de Biotechnologie des Plantes, UMR CNRS 8618, Bâtiment 630, Université Paris-Sud, 91405 Orsay Cedex, France*
[b] *IBBMC, UMR CNRS 8619, Bâtiment 430, Université Paris-Sud, 91405 Orsay Cedex, France*

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Abstract The activation pathway of the chloroplastic NADP-dependent malate dehydrogenase (MDH) by reduced thioredoxin has been examined using a method based on the mechanism of thiol/disulfide interchanges, i.e. the transient formation of a mixed disulfide between the target and the reductant. This disulfide can be stabilized when each of the partners is mutated in the less reactive cysteine of the disulfide/dithiol pair. As NADP-MDH has two regulatory disulfides per monomer, four different single cysteine mutants were examined, two for the C-terminal bridge and two for the N-terminal bridge. The results clearly show that the nucleophilic attack of thioredoxin on the C-terminal bridge proceeds through the formation of a disulfide with the most external Cys377. The results are less clear-cut for the N-terminal cysteines and suggest that the Cys24–Cys207 disulfide bridge previously proposed to be an intermediary step in MDH activation can form only when the C-terminal disulfide is reduced.

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

1. Introduction

The thioredoxin (TRX)-mediated redox activation of the chloroplastic NADP-dependent malate dehydrogenase (NADP-MDH; EC 1.1.1.82) proceeds through the reduction of two disulfides per monomer (the enzyme is a homodimer), namely, the N-terminal C24–C29 disulfide and the C-terminal C365–C377 disulfide [1]. These two disulfides are clearly visible in the X-ray structures available now for the inactive, oxidized forms of the enzymes from sorghum [2] and *Flaveria* [3]. A transient isomerization of the N-terminal disulfide into a C24–C207 disulfide has also been proposed [4]. A poorly understood feature of the activation mechanism is its strict requirement for reduced TRX: the chemical dithiol reagent dithiothreitol (DTT) is quite inefficient, unless used at very high concentrations and in very special conditions (high ionic strength) [5], whereas its redox potential (−330 mV) is quite adequate for disulfide reduction: the redox potentials of the individual disulfides of NADP-MDH ranging between −330 mV for the C-terminal disulfide and −280 mV for the N-terminal disulfide [6]. Classically, TRX reduction of disulfides is believed to occur by a two-step mechanism [7]: first, a nucleophilic attack on the most reactive cysteine of the target disulfide by the most reactive cysteine of reduced TRX (the most N-terminal one) to form a mixed disulfide, then the disruption of the disulfide by the second cysteine of TRX. By using single cysteine mutants of each of the partners under oxidizing conditions, a stable mixed disulfide can be formed between TRX and its target, provided that the remaining cysteines are those being primarily involved in the formation of the mixed disulfide. This approach has been successfully used in several cases to identify the reactive cysteines of various TRX targets [8–13]. We used this approach recently [14] to show that a mixed disulfide can be formed between TRX and the internal Cys207 of NADP-MDH when the N-terminal disulfide is open, and this finding substantiated the hypothesis that such a mixed disulfide could be formed transiently during the activation process.

In the present study, we further investigated the interaction between the regulatory disulfides of NADP-MDH and TRX, still using the mixed-disulfide approach, in order to identify, in each of the two disulfides initially present in the oxidized enzyme, which cysteine is engaged in the mixed-disulfide intermediate formed as a first step of the activation process. This identification can provide useful information about the interaction mechanism between TRX and the target disulfides. It also constitutes a first step towards the isolation of a mixed-disulfide-stabilized complex between TRX and one of its targets. Such a stable complex could be used for structural studies. An X-ray structure of the complex would indeed allow to solve the conformational effect of TRX on one of its targets.

2. Materials and methods

2.1. Production of mutant proteins

All of the mutant cDNAs have been obtained previously, either by PCR for the introduction of the C39S mutation into the pET-TRX h-type cDNA [15], or by the method of Kunkel [16] for MDH mutants. In the C24S and C29S MDH mutants, Cys24 and -29, respectively, have been replaced by serines [17]. In the C365A and C377A MDH mutants, Cys365 and -377, respectively, have been replaced by alanines [18]. In the C29S-2C MDH mutant, Cys29 has been replaced by serine and Cys365 and -377 (2C) have been replaced by alanines [4]. All these cDNAs were cloned into pET-8c vectors and used to transform *Escherichia coli* strain BL21 (DE3) [19].

*Corresponding author. Fax: (33)-1-69 15 34 23.
E-mail address: miginiac@ibp.u-psud.fr (M. Miginiac-Maslow).

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, thiobis(2-nitrobenzoic acid); DTT, dithiothreitol; NADP-MDH, NADP-dependent malate dehydrogenase; TRX, thioredoxin

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2.2. Purification of the recombinant proteins

The expressed proteins were purified to homogeneity by previously described procedures. The purification of Trx combined heat shock, ammonium sulfate fractionation, exclusion chromatography on Sephadex G50 and ion-exchange chromatography on DEAE-Sephacel [14]. The NADP-MDH was purified combining ammonium sulfate fractionation, DEAE-Sephacel and affinity chromatography on Matrex RedA [17,18].

2.3. Polyacrylamide gel electrophoresis (PAGE)

Denaturing (4% (w/v) SDS) electrophoresis was carried out on 10% polyacrylamide gel. Reductant was omitted, in order to avoid the disruption of mixed disulfides. Gels were stained with Coomassie blue (2.5 g/l).

2.4. Immunoblot analysis

MDH was immunodetected using a rabbit polyclonal antibody raised against recombinant sorghum NADP-MDH. Proteins were electrophoresed and transferred to nitrocellulose membranes [20]. Proteins were detected using primary antibody, goat anti-rabbit IgG coupled to peroxidase (Sigma), and visualized by chromogenic reaction with 4-chloro-1-naphthol.

2.5. Mixed-disulfide formation between MDH and Trx by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) oxidation

2.5.1. Preparation of the mixed-disulfide MDH–TNB (thiobis(2-nitrobenzoic acid)) and C39S-TNB. The method described by Wang et al. [8] was used. The proteins were reacted with a 20-fold excess of DTNB and the reaction was monitored at 412 nm until completion. The reaction mixtures were then dialyzed against Tris–HCl 30 mM, pH 7.9, buffer using Amicon Centricon filtration units to remove excess of DTNB and TNB. The quantification of TNB-derivatized proteins was performed by reaction of an aliquot of TNB-derivatized proteins with a 100-fold excess of DTT and monitoring the release of TNB at 412 nm. The concentration of TNB was calculated by using an extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm. As some of the mutants (especially after a period of storage) tended to form dimers or polymers, the proteins were treated with 1 mM DTT, then dialyzed in Centricon concentrators, unless otherwise specified, before the DTNB derivatization. This treatment did not activate the enzymes which required reduced TRX for activity.

2.5.2. Preparation of the mixed disulfides between MDH and Trx C39S. TNB-derivatized MDH was reacted with a three-fold molar excess (per subunit) of Trx C39S. The same molar ratio was used for the reaction between TNB-derivatized Trx and MDH. The completion of the reaction was monitored at 412 nm for the release of TNB.

2.6. NADP-MDH activity measurements

The enzymes were pre-activated by incubation with 20 μM recombinant TRX h from *Chlamydomonas* and 10 mM DTT in 100 mM Tris buffer, pH 7.9. The activity was measured on aliquots added to a spectrophotometer cuvette containing 1 ml of reaction medium (210 μM NADPH, 780 μM OAA in 100 mM Tris buffer, pH 7.9). The activity was measured by the decrease in absorbance at 340 nm due to NADPH oxidation.

3. Results and discussion

In our previous study [14], we presented evidence that in plant TRXs, the primary nucleophile is the most N-terminal cysteine, exactly like in *E. coli* and human TRXs. Accordingly, for the present study, we used a mutant TRX where the second cysteine was substituted for a serine (C39S mutant) and the most N-terminal cysteine (C36) retained. As in our previous study, we took advantage of the relative lack of specificity of NADP-MDH for various TRX types to use the h-type TRX from the green alga *Chlamydomonas* which features only two cysteines in its primary sequence: those belonging to the active site. This peculiarity excludes the risk of making artifactual mixed disulfides with the additional cysteines present in Trx m and f sequences [21].

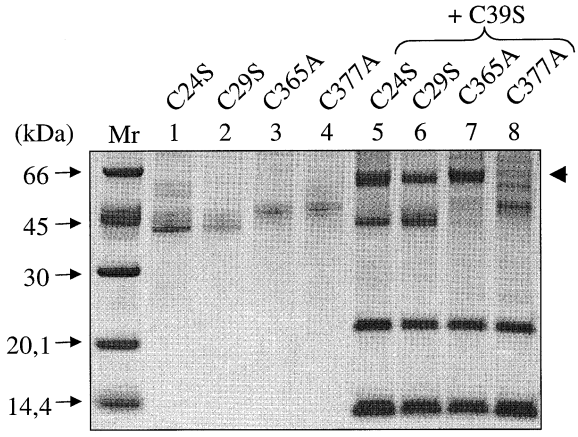

Fig. 1. Formation of mixed disulfides between MDH mutated at N- or C-terminal cysteines and C39S Trx h. MDH–Trx reaction mixtures were analyzed by non-reducing SDS–PAGE. All MDH proteins were pre-treated with 1 mM DTT, then dialyzed and derivatized with DTNB before incubation with C39S Trx h. The arrow indicates MDH–Trx complexes.

In order to distinguish which cysteine in each regulatory disulfide is the one forming a mixed disulfide with TRX, various single cysteine mutant NADP-MDHs had to be used. All of them were engineered previously and their activation properties have been studied [4,17,18].

In the literature, two major procedures are used to create mixed disulfides: either mix the mutant TRX with the mutant target in the presence of copper ions, or derive one of the partners with DTNB and allow it to react with the other. When the mixed disulfide forms, TNB-ion is released and can be monitored by its absorbance at 412 nm. This allows to follow the formation of the complex in a time-dependent manner and to determine which of the two cysteines of the mixed disulfide is the primary nucleophile. Indeed, the efficiency of the reaction, hence the amount of complex formed, depends on which of the partner proteins is derivatized. For all these reasons, we chose the DTNB derivatization method to address the problem of the interaction of TRX with the regulatory disulfides of NADP-MDH.

A problem often encountered when only one cysteine of a disulfide is mutated is a tendency of the mutant to dimerize by

Table 1
Relative amounts of free MDH or MDH in complex with Trx

MDH mutant used in reaction mixture	Relative amount of protein (%)	
	MDH	MDH–Trx complex
C24S	43 ± 1	57 ± 1
C29S	55 ± 3	45 ± 3
C377A	64 ± 2	36 ± 2
C365A	14 ± 12	86 ± 12

Quantification of amounts of proteins was performed by densitometric scans (Masterscan, Scanalytics, CSPI).

forming inter-subunit disulfides, especially upon storage. To avoid this problem, all the mutant proteins were pre-treated with 1 mM DTT, then dialyzed prior to the DTNB treatment. This pre-treatment did not lead to any activation of the MDH mutants.

In a first set of experiments, we used four mutant MDHs (C24S, C29S, C365A and C377A) lacking only one of the cysteines of either the N-terminal or the C-terminal regulatory disulfide. An attempt to get mixed-disulfide-linked complexes between these mutants and TNB-derivatized Trx C39S was totally unsuccessful: none of the remaining single cysteines was able to attack the disulfide bond between Trx and TNB (data not shown). In contrast, when Trx C39S was mixed with DTNB-treated MDH mutants, 54 kDa heterodimers could be observed with some of the mutants (Fig. 1) on denaturing non-reducing polyacrylamide gels, corresponding to covalent coupling of one MDH subunit (42 kDa) and one Trx molecule (12 kDa). No 54 kDa species were observed on reducing gels (data not shown), thus, it can be concluded that the heterodimers were linked by disulfide bridges. We have previously shown that the oxidized wild-type (WT) protein does not show dimers on non-reducing gels and forms only very small amounts (ca. 10%) of heterodimers with single cysteine TRX mutants [14]. In contrast, the MDH C365A mutant, where the most internal cysteine of the C-terminal disulfide is missing, is almost totally under a heterodimeric (54 kDa) form (Fig. 1, lane 7 and Table 1). This is not the case for the MDH mutated on the other C-terminal cysteine (C377A mutant). This one remains predominantly (ca. 80%) a monomer (Fig. 1, lane 8 and Table 1). This clearly demonstrates that TRX's nucleophilic attack occurs on the most external cysteine of the C-terminal disulfide. The result is much less clear-cut with the MDHs mutated on the cysteines of the N-terminal disulfide bridge. Indeed, both the C24S and C29S mutants were

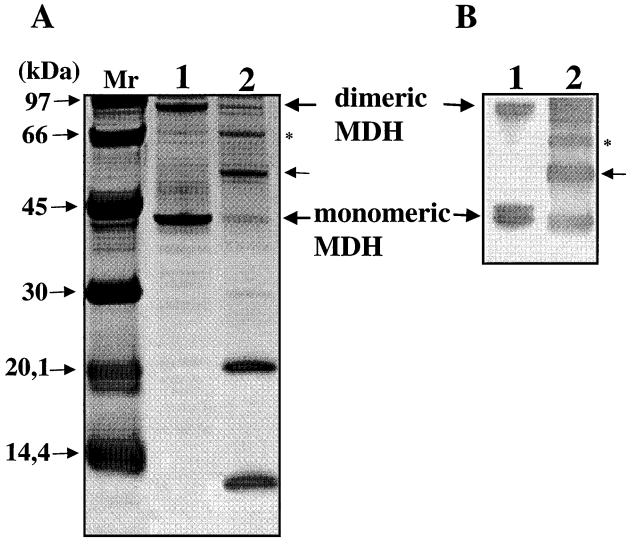

Fig. 2. Formation of mixed disulfides between C29S/C365A/C377A MDH and C39S Trx h. MDH–Trx reaction mixtures were separated by non-reducing SDS–PAGE, then (A) proteins were visualized by staining with Coomassie blue or (B) analyzed by Western blot using an antibody against NADP-MDH. MDH was pre-treated with 1 mM DTT, then dialyzed and derivatized with DTNB before incubation with C39S Trx h. The arrow indicates a complex between one MDH monomer and one TRX, and the asterisk indicates one MDH monomer complexed with two TRX molecules.

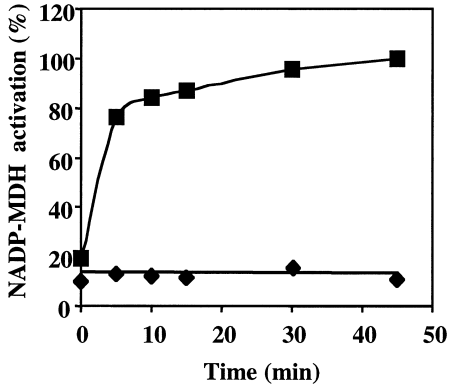

Fig. 3. Activation kinetics of C29S/C365A/C377A MDH by DTT alone (closed diamonds) or DTT and Trx (closed squares). MDH was activated by incubation in the presence of 10 mM DTT with or without 17 μM Trx h in 100 mM Tris–HCl buffer, pH 7.9. The activity was measured on aliquots and is expressed as percent of the activity of the fully activated C29S/C365A/C377A MDH.

able to form important proportions of heterodimers, with a slight advantage of the C24S mutant (82%) over the C29S mutant (72%). This result was rather unexpected. First, it suggested that TRX showed no preference for a specific N-terminal cysteine. Second, in view of previous mutagenesis results [4], showing the existence of an intermediary disulfide linking Cys24 and Cys207, one could expect that this bridge was present in the C29S mutant and thus, that no heterodisulfide could be formed with TRX, unless Cys36 of TRX could disrupt it and form a stable disulfide with one of the target's cysteines. Keeping in mind that the presence of a Cys24–Cys207 disulfide had been demonstrated using a mutant devoid not only of Cys29 but also of the C-terminal disulfide, the complex-forming ability of the C29S/C365A/C377A mutant was examined. Fig. 2 (A and B) shows that in non-reducing gels, this MDH mutant is almost equally distributed between two major protein bands: one at 42 kDa, corresponding to the usual monomeric form, the other at ca. 84 kDa, corresponding to a MDH dimer (lane 1). Upon incubation with C39S TRX, the bands corresponding to both forms were markedly decreased and two new bands appeared: one at 54 kDa, corresponding to one MDH monomer complexed with one TRX, and another one at 66 kDa, corresponding to one MDH monomer complexed with two TRX molecules (lane 2). The fact that upon addition of the C39S TRX, the dimeric MDH form was strongly decreased and heteromeric complexes with Trx appeared, suggests that the disulfide bridge present in the oxidized form is disrupted by Cys36 of TRX and that a stable mixed disulfide is formed in this case. This property of TRXs is not unusual and has been successfully used to trap some TRX targets, for instance a TRX-dependent peroxidase, where the TRX-reducible disulfide cross-links two subunits [12]. The presence of MDH dimers on non-reducing gels has never been reported before. We observed it only for the C29S/C365A/C377A mutant. A Cys24–Cys24 bridge between the monomers of this mutant is highly unlikely: the C29S/C207A/C365A/377A mutant, where Cys24 is the only available cysteine, is constitutively active when freshly isolated. Upon ageing, it can be partially inactivated, but then the addition of 1 mM DTT directly to the reaction cuvette restores its activity, whereas reduced TRX (in the absence of DTT) has no effect at all [4].

The C29S/C365A/377A, on the contrary, needs reduced TRX for activation (Fig. 3), and redox titrations showed that it had a titratable disulfide bridge with a rather electronegative (−310 mV) redox potential [6]. These results support the assumption that the observed dimers are cross-linked by a TRX-reducible disulfide bridge linking Cys24 of one subunit to Cys207 of the other subunit. This assumption correlates pretty well with structural data showing that Cys207 of one subunit is closer to Cys24 of the other subunit than to Cys24 of its own subunit [2]. The presence of an inter-subunit disulfide has also been predicted by Carr et al. [3] based on the structure of *Flaveria bidentis* NADP-MDH. As discussed by these authors, the disruption of this disulfide would require an attack of TRX on Cys203, the *Flaveria* equivalent of Cys207, previously shown to be able to form mixed disulfides with TRX [14]. After the disruption of the putative Cys24–Cys207 inter-subunit disulfide, a complex is formed between TRX and one or both of the cysteines of the disulfide, as shown by the presence of two different complexes: one at 54 kDa and one at 66 kDa. A not easily explainable observation is the persistence of a monomeric form of this mutant, unless an equilibrium between monomeric and dimeric forms exists. The C29S single mutant showed practically no dimeric form, hence no cross-link between subunits. This would suggest that the Cys24–Cys207 disulfide forms only in mutants having no C-terminal disulfide. Transposed to the case of the WT enzyme, this observation suggests that the reduction of the C-terminal disulfide would precede the formation of the putative C24–C207 disulfide during the activation process. Nuclear magnetic resonance experiments have shown that the C-terminal extension of NADP-MDH is released from the active site upon reduction of the C-terminal disulfide bridge [22]. This release might be a pre-requisite for the formation of the C24–C207 disulfide.

In conclusion, our results show unambiguously that TRX reduces the C-terminal disulfide of NADP-MDH via a nucleophilic attack on its most external Cys377. This result is consistent with structural data showing that this cysteine is exposed to the solvent [2]. Concerning the events occurring at the N-terminus, the results are less clear-cut. Structural data suggest that Cys29 is the one attacked first. Mixed-disulfide data show that all three potential partner cysteines are able to interact with TRX. It is possible that mutation of one of the N-terminal cysteines (24 or 29) modifies the reactivity of the other one. From a mechanistic point of view, it appears clearly that single cysteine mutant TRX is able to disrupt inter-subunit disulfides, but not intra-subunit disulfides of NADP-MDH: the dimeric MDH form of MDH disappears upon treatment with the TRX mutant, but almost no complex is formed with WT MDH where all cysteines are paired in disulfide bridges [14]. The reason might be the proximity of the second cysteine of the intramolecular disulfides, able to attack the mixed disulfide and disrupt it, whereas the disruption of an inter-subunit disulfide is followed by the physical separation of the second cysteine from the newly formed mixed disulfide. This observation is in line with the recent use of single cysteine mutant TRXs as a bait for target proteins, resulting in preferential trapping of a peroxiredoxin whose reaction mechanism is based on the reduction of an inter-subunit disulfide [12].

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