FEBS 21525 FEBS Letters 444 (1999) 165–169

The internal Cys-207 of sorghum leaf NADP-malate dehydrogenase can form mixed disulphides with thioredoxin

Aymeric Goyer, Paulette Decottignies, Stephane Lemaire, Eric Ruelland, Emmanuelle Issakidis-Bourguet, Jean-Pierre Jacquot, Myroslawa Miginiac-Maslow*

Institut de Biotechnologie des Plantes, UMR 8618 CNRS, Bâtiment 630, Université de Paris-Sud, 91405 Orsay Cedex, France

Received 30 November 1998; received in revised form 7 January 1999

Abstract The role of the internal Cys-207 of sorghum NADP-malate dehydrogenase (NADP-MDH) in the activation of the enzyme has been investigated through the examination of the ability of this residue to form mixed disulphides with thioredoxin mutated at either of its two active-site cysteines. The *h*-type *Chlamydomonas* thioredoxin was used, because it has no additional cysteines in the primary sequence besides the active-site cysteines. Both thioredoxin mutants proved equally efficient in forming mixed disulphides with an NADP-MDH devoid of its N-terminal bridge either by truncation, or by mutation of its N-terminal cysteines. They were poorly efficient with the more compact WT oxidised NADP-MDH. Upon mutation of Cys-207, no mixed disulphide could be formed, showing that this cysteine is the only one, among the four internal cysteines, which can form mixed disulphides with thioredoxin. These experiments confirm that the opening of the N-terminal disulphide loosens the interaction between subunits, making Cys-207, located at the dimer contact area, more accessible.

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

1. Introduction

NADP-dependent malate dehydrogenase (EC 1.1.1.82) is known to be activated by thiol/disulphide interchange with reduced thioredoxin (Trx), a process dependent on the photosynthetic electron transfer in chloroplasts [1]. The enzyme has no activity at all in the oxidised form. The reduction of three different disulphides has been shown to play a role in the activation process: a C-terminal one (Cys-365–Cys-377), an N-terminal one (Cys-24–Cys-29) and possibly a disulphide linking Cys-24 and Cys-207 whose formation has been proposed as an intermediary step in the activation mechanism [2].

Whereas the link between the Trx-dependent reduction of these disulphides and the activation of the enzyme is well established, the information about the interchange thiols forming the transient mixed disulphides which constitute the first step of a disulphide reduction process is still missing. In particular, the recent identification of a Cys-24–Cys-207 disulphide in a mutant MDH missing Cys-29 [2] raises the question of the accessibility of Cys-207 to Trx. Indeed, molecular modelling indicated that this cysteine is situated at the dimer contact area of the homodimeric enzyme, hence is probably not freely accessible [3]. The formation of a stable mixed disulphide between an NADP-MDH truncated at the N-terminus and labelled cysteine in solution has been reported [4]. The truncated enzyme is devoid of the N-terminal extension which bears the N-terminal regulatory disulphide but retains the C-terminal disulphide and the four internal cysteines including Cys-207. The mixed disulphide must then be formed with one of the four internal cysteines of the protein which should be available and reactive. The mixed disulphide approach has recently been used to identify the interacting cysteines in several partner enzymes of Trx: Trx reductase [5], phosphoribulokinase [6], the target peptide of the NF-κB transcription factor [7] and a synthetic model peptide [8]. In each case, single cysteine mutants of Trx and its target were created, and a stable mixed disulphide could be formed in the presence of an oxidant if the remaining cysteine of each of the partners was sufficiently available and reactive.

We used the mixed disulphide strategy to investigate whether the internal Cys-207 of NADP-malate dehydrogenase was able to form a stable complex with Trx mutated in one of its active-site cysteines. Classically, NADP-MDH is believed to be activated by the chloroplastic Trx *m* [9]. In fact, it was later demonstrated that it can be activated by the chloroplastic Trx *f* with an even better efficiency [10,11], but also by the cytosolic Trx *h*, either from higher plants [12] or from *Chlamydomonas* [13,14]. We took advantage of this lack of specificity of NADP-MDH towards the Trx source to engineer Trx *h* from *Chlamydomonas reinhardtii* mutated in either of its active-site cysteines. Then we investigated the ability of these mutants to form mixed disulphides with NADP-MDH. Indeed, in Trx *h*, there are only two Cys in the primary sequence: those belonging to the active-site disulphide [13]. Using this Trx excludes the possibility of getting artefactual disulphides with the additional cysteines present in Trx *m* and *f* sequences [9]. The complex-forming ability of these mutants was tested first with the N-terminally truncated form of NADP-MDH, then with an enzyme either having retained its N-terminal cysteines (Δ-15 MDH), or mutated in these cysteines (C24S/C29S mutant, abbreviated NDM, for N-terminal double mutant) to mimic the situation where the disulphide is reduced. Proteins (full-length or deleted) mutated in Cys-207 were used as controls.

*Corresponding author. Fax: (33) (1) 69 33 64 23.
E-mail: miginiac@ibp.u-psud.fr

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio(2-nitrobenzoic acid); DTT, dithiothreitol; NADP-MDH, NADP-dependent malate dehydrogenase; Trx, thioredoxin; Δ-15 MDH, MDH with the 15 most N-terminal residues deleted; ΔN MDH, MDH with the 33 most N-terminal residues deleted; NDM MDH, MDH mutated at the two most N-terminal cysteines 24 and 29

PII: S0014-5793(99)00051-4

2. Materials and methods

2.1. Production of WT and mutant proteins

The plasmids used for mutagenesis were the pET-Trx *h* and pET-MDH constructions described previously [2,3,14,15]. PCR was used to introduce site-directed mutations into the WT Trx *h* cDNA. The oligonucleotides used were as follows: C36S Up: 5′-TCACTGCTACGTGGAGCGGTCCTTGCAAG-3′; C36S Down: 5′-CTTGCAAGGACCGCTCCACGTAGCAGTGA-3′; C39S Up: 5′-CGTGGTGCGGTCCTAGCAAGATGATTGCGC-3′; C39S Down: 5′-GCGCAATCATCTTGCTAGGACCGCACCACG-3′.

The NADP-MDH ΔN, C207A, NDM and Δ-15 proteins were created previously [2,3,14,15]. The ΔN mutant [15] lacks the portion of the N-terminal extension bearing the regulatory disulphide (the 33 most amino-terminal residues), the Δ-15 mutant lacks the 15 most amino-terminal residues, but retains the regulatory (Cys-24–Cys-29) disulphide. This mutant is shortened by the portion of the enzyme which undergoes spontaneous proteolysis. It has all the biochemical characteristics of the full-length WT enzyme but presents the advantages of yielding much more homogeneous preparations and of avoiding multiple bands or smears on polyacrylamide gels (unpublished). In the NDM (N-terminal double mutant) Cys-24 and -29 have been replaced by serines [15]. The ΔN.C207A mutant was derived from the ΔN and C207A [15] mutants. The pET-MDH vectors were digested with *Esp*I and the resulting C207A fragment was ligated to *Esp*I-digested pET-ΔN MDH.

The resulting constructions were used to transform *Escherichia coli* strain BL21 (DE3) [16].

2.2. Purification of the recombinant proteins

The expressed proteins were purified to homogeneity by previously described procedures [2,10,15]. The purification of Trx combined heat shock, ammonium sulphate fractionation, exclusion chromatography on Sephadex G50 and ion-exchange chromatography on DEAE-Sephacel. The NADP-MDH was purified combining ammonium sulphate fractionation, DEAE-Sephacel and affinity chromatography on Matrex RedA.

2.3. Assay for NADP-MDH activity

The enzymes were preactivated by incubation in the presence of 10 mM DTT and 17 or 50 μM Trx *h* in 100 mM Tris-HCl buffer, pH 7.9. The activity was measured on aliquots added to a spectrophotometer cuvette containing 1 ml of reaction medium (210 μM NADPH, 750 μM OAA in 100 mM Tris-HCl buffer, pH 7.9). The activity was estimated by the decrease in absorbance at 340 nm.

2.4. Polyacrylamide gel electrophoresis

Denaturing (4% (w/v) SDS) electrophoresis was carried out on 10% polyacrylamide gel. Gels were stained with Coomassie blue (2.5 g/l).

2.5. Mixed disulphide formation between MDH and Trx by cupric ion-catalysed oxidation

Mixtures of selected mutants were prepared at a 3:1 molar ratio of Trx:MDH subunit and incubated at 4°C in 100 mM Tris-HCl buffer pH 7.9, 150 mM NaCl and 20% glycerol in the presence of 2 mM $CuSO_4$ for 3 h; the final concentration of MDH was 0.4 mg/ml.

2.6. Mixed disulphide formation between MDH and Trx by DTNB oxidation

2.6.1. Preparation of the mixed disulphide ΔN-TNB, C39S-TNB and C36S-TNB. The method described by Wang et al. [5] 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 dialysed against Tris-HCl 30 mM, pH 7.9 buffer using Amicon Centricon filtration units to remove excess DTNB and TNB. The quantitation of TNB-derivatised proteins was performed by reaction of an aliquot of TNB-derivatised proteins with a 100-fold excess of DTT and monitoring the release of TNB at 412 nm. The concentration of TNB was calculated using an extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm.

2.6.2. Preparation of the mixed disulphides between ΔN MDH and Trx C39S or C36S. ΔN-TNB MDH was reacted with a 3-fold molar excess of Trx C39S or C36S. The same molar ratio was used for the reaction between TNB-derivatised Trx and ΔN MDH. The reaction was monitored at 412 nm for the release of TNB and quantitated.

2.7. Reverse phase HPLC

An aliquot of complex-forming mixture diluted in 0.1% trifluoroacetic acid was injected into a column (Vydac, 300 Å, 4.6×250 mm) previously equilibrated with 38% acetonitrile in 0.1% trifluoroacetic acid. The elution was performed with a 30 min linear gradient of 38–60% acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 1 ml/min.

2.8. N-terminal sequencing

Proteins were sequenced with an Applied Biosystems (Perkin Elmer) model 476A automated sequencer equipped with an on-line phenylthiohydantoin amino acid analyser.

3. Results

3.1. Formation of mixed disulphides between ΔN MDH and Trx h mutated at active site cysteines

Mixtures of Trx *h* and ΔN MDH were incubated in the presence of the oxidant $CuSO_4$, then analysed by SDS-PAGE in non-reducing conditions (Fig. 1). In mixtures containing ΔN MDH and either C39S Trx *h* or C36S Trx *h* (lanes 1 and 3), a 50 kDa species was prominent, consistent with covalent coupling between one ΔN MDH (38 kDa) subunit and one molecule of Trx (12 kDa). When ΔN MDH was devoid of Cys-207 (lanes 2 and 4), this 50 kDa species was absent, indicating that Cys-207 was implicated in coupling between MDH and Trx. When DTT was added to C39S Trx/ΔN MDH or to C36S Trx/ΔN MDH reaction mixtures (lanes 5 and 7), only separate Trx and MDH bands were observed, thus demonstrating that the two proteins were covalently bonded via a mixed disulphide. Under non-reducing conditions, two other species were revealed in C39S/ΔN or C36S/ΔN mixtures (lanes 1 and 3): the 24 kDa species represents the dimeric form of single cysteine Trx mutant that did not form with WT Trx (data not shown). The 76 kDa species, which is missing when Cys-207 is mutated (lanes 2 and 4), represents the dimeric form of MDH. These species are not present under reducing conditions (lanes 5 and 7). These observations provide evidence that Cys-207 of MDH and the unique active-site Cys of both Trx mutants are implicated in

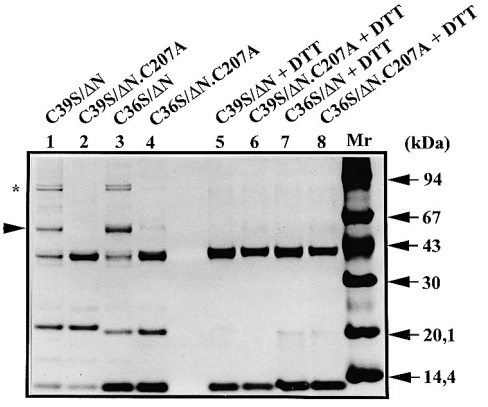

Fig. 1. Formation of mixed disulphides between ΔN MDH and Trx *h* mutated at active site cysteines by cupric ion-catalysed oxidation. ΔN MDH/Trx reaction mixtures were analysed by non-reducing SDS-PAGE. Proteins were incubated in the presence of 2 mM $CuSO_4$ (lanes 1–4) or in the presence of 0.1 M DTT (lanes 5–8) for 3 h. The arrow indicates MDH/Trx complexes and asterisks dimeric MDH.

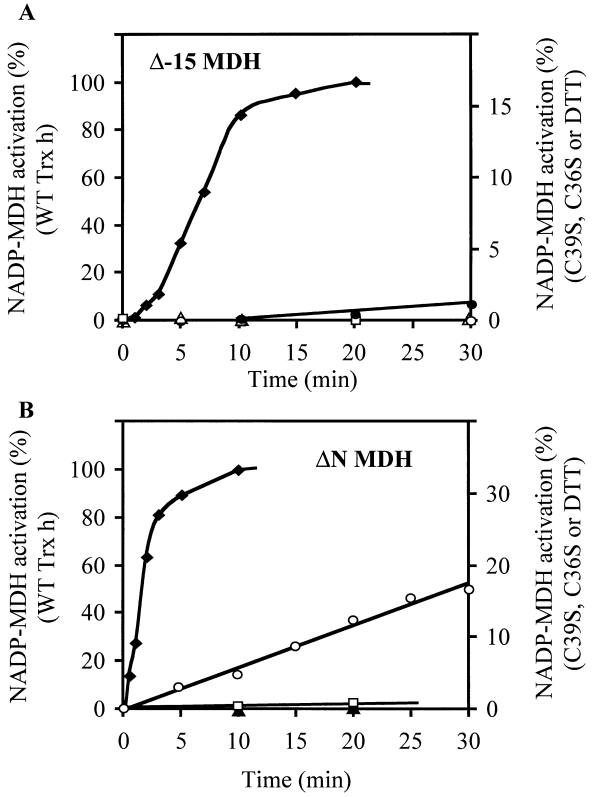

Fig. 2. Activation kinetics of Δ-15 MDH (A) or ΔN MDH (B) by 17 μM WT Trx *h* (◆), 17 μM C39S (○), 17 μM C36S (▲), 50 μM C39S (●), 50 μM C36S (△) and DTT alone (□). MDHs were preactivated by incubation in the presence of 10 mM DTT and 17 or 50 μM Trx *h* in 100 mM Tris-HCl buffer, pH 7.9. The activity was measured on aliquots and is expressed as % of the activity of the fully activated Δ-15 MDH (A) or ΔN MDH (B).

the formation of dimeric MDH or Trx and suggest a reactivity of these cysteines.

3.2. Quantification of Trx h/ΔN MDH mixed disulphide formation using DTNB method

The cupric ion complex-forming method is convenient to visualise the Trx/MDH complexes, but it does not allow a precise quantification of the amount of complex formed. To overcome this problem, we have used a method based on DTNB oxidation of thiols [5]. Moreover, this technique was also used to determine which Cys (Cys-207 of MDH or the unique remaining active-site Cys of both Trx mutants) is the primary nucleophile for the complex formation. The results (Table 1) demonstrate that 50% of TNB-derivatised ΔN MDH formed a complex with either C39S Trx or C36S Trx. In contrast, when C39S Trx or C36S Trx was derivatised with TNB, 17% and 20% respectively were able to form a complex with ΔN MDH. These results show no difference in the complex-forming ability of C39S and C36S Trx mutants, which is

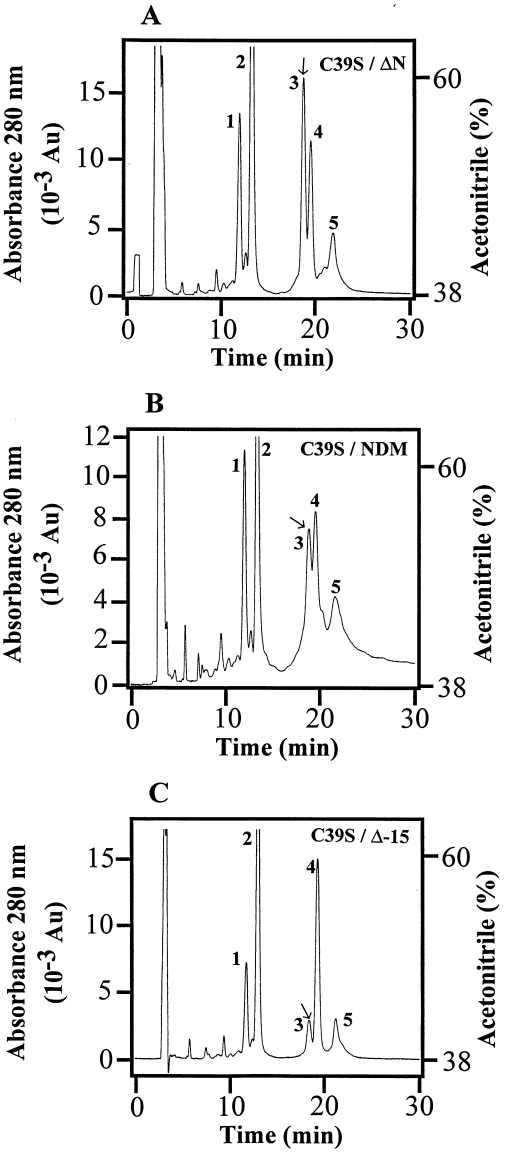

Fig. 3. RP-HPLC analyses of cupric ion-catalysed complex-forming reaction mixtures of C39S Trx h and respectively ΔN (A), NDM (B) or Δ-15 (C) MDHs. MDH/C39S complexes are indicated by the arrowhead.

consistent with the results obtained with cupric oxidation. However, when TNB-derivatised ΔN MDH was used instead of TNB-derivatised Trx *h* mutants, larger amounts of complexes were formed. This result provides evidence that the unique active-site Cys of both Trx mutants was more efficient in performing a nucleophilic attack than Cys-207.

3.3. Identification of the primary nucleophile of Trx h

Brandes et al. [6] have identified Cys-46 as the primary

Table 1
Formation of Trx/MDH complexes using the DTNB method

Reaction mixtures	C39S Trx/ΔN-TNB MDH	C36S Trx/ΔN-TNB MDH	C39S-TNB Trx/ΔN MDH	C36S-TNB Trx/ΔN MDH
TNB-derivatised proteins engaged in a mixed disulphide (%)	50	50	17	20

nucleophile of Trx *f* and have shown that only C49S Trx *f* mutant was able to form a complex with a C16S phosphoribulokinase mutant. In contrast, in the present study, both single cysteine Trx *h* mutants were able to form a mixed disulphide complex with ΔN MDH. To determine which Cys of Trx *h* is the primary nucleophile, activation kinetics of NADP-MDH were performed. Only WT Trx was able to activate Δ-15 MDH very efficiently (Fig. 2A). The C39S Trx mutant slightly activated the enzyme when used at a high concentration (50 μM) whereas the C36S Trx mutant was totally unable to activate the enzyme. The ΔN MDH activation was then tested (Fig. 2B). This MDH is activated more rapidly than Δ-15 MDH, because of the absence of the Cys-24–Cys-29 or Cys-24–Cys-207 bridges, the reduction of which constitutes the rate-limiting step of the activation process [2,15]. Here again, only the C39S Trx mutant was able to activate ΔN MDH. But in this case, lower concentrations of Trx (17 μM) yielded appreciable activation rates. A similar result was also obtained with other MDH disulphide bridge mutants (data not shown), providing evidence that Cys-36 is the primary nucleophile of Trx *h*. For this reason, we have used only the C39S mutant in further experiments.

3.4. Formation of mixed disulphides using NDM and Δ-15 MDHs

ΔN MDH, devoid of its N-terminal extension, is monomeric [15]. Thus, Cys-207 should be easily accessible. In contrast, WT MDH is dimeric and very compact in its oxidised form [1]. The N-terminal bridge present in this oxidised form

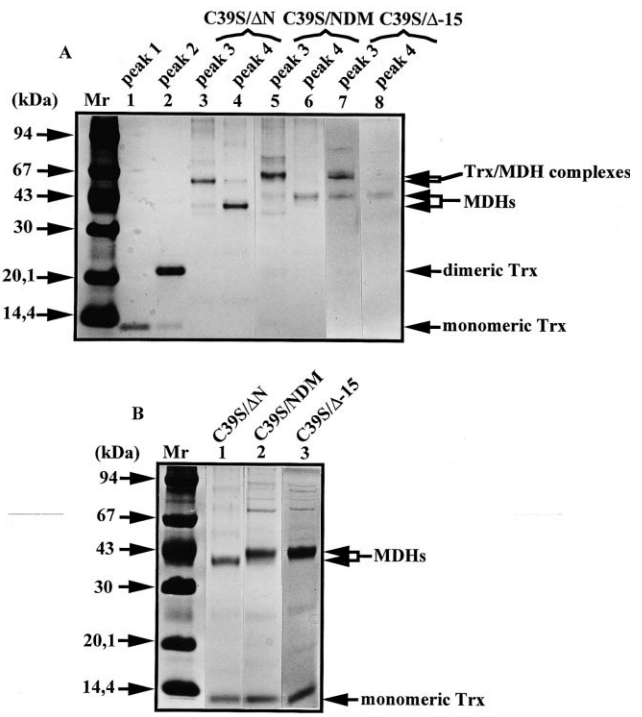

Fig. 4. Polyacrylamide gel electrophoresis of peaks after separation by RP-HPLC. A: Denaturing electrophoresis was carried out in non-reducing conditions. The patterns corresponding to peaks 1 and 2 were identical whatever the reaction mixture. B: Peaks 3 were analysed in reducing conditions by treatment with 15 mM DTT. MDHs and MDH/Trx complexes are indicated by two arrowheads because the full-length MDH and the deleted MDH have different molecular masses.

Table 2
Relative amounts of free MDH or MDH in complex with Trx as separated by RP-HPLC experiments

Reaction mixture	Relative amount of protein (%)	
	MDH	MDH-Trx complex
Δ-15 MDH/C39S Trx	89 ± 1	11 ± 1
NDM MDH/C39S Trx	56 ± 1	44 ± 1
ΔN MDH/C39S Trx	46 ± 6	54 ± 6

The values are the means ± S.E.M. from three experiments. The relative quantities were calculated by integrating areas of MDH and complex peaks using the Borwin program.

is the Cys-24–Cys-29 disulphide. Then, Cys-207 should be in a reduced form, but poorly accessible. In the NDM mutant (Cys-24 and Cys-29 replaced by Ser), which mimics the situation where the Cys-24–Cys-29 bridge has been reduced, the interaction between MDH subunits is weakened [1] and Cys-207 should be more accessible. To test this hypothesis, a comparison of the abilities of Δ-15, NDM and ΔN MDHs to form complexes with Trx *h* C39S has been investigated. The Δ-15 form was used instead of the full-length WT MDH in a search for homogeneity (see Section 2), its properties being identical to those of the full-length protein.

The ultimate goal of the study being the possibility of isolating mixed disulphide complexes between MDH and Trx for further biochemical and structural studies, the reaction mixtures were analysed by RP-HPLC. In the HPLC profiles of C39S Trx/ΔN MDH, C39S Trx/NDM MDH and C39S Trx/Δ-15 MDH mixtures, five peaks (1, 2, 3, 4 and 5) were observed (Fig. 3). The protein composition of the peaks was examined by SDS-PAGE in non-reducing conditions (Fig. 4). Peak 1 contained the monomeric form of Trx (lane 1), peak 2 the dimeric form of Trx (lane 2), peak 4 contained MDH (lanes 4, 6 and 8) and peak 5 was heterogeneous (data not shown). In all cases, peak 3 revealed a higher molecular mass species consistent with a coupling between one Trx molecule and one MDH subunit (lanes 3, 5 and 7). Analysis of peak 3 by N-terminal sequencing revealed the presence of only MDH and Trx N-terminal sequences (data not shown). In MDH mutants where Cys-207 was substituted (C207A or C207A/ΔN), peak 3 was absent (data not shown), confirming that Cys-207 is implicated in the complex formation. To confirm that MDH and Trx were linked by a mixed disulphide, the proteins eluted in peak 3 were treated with DTT then analysed by SDS-PAGE (Fig. 4B, lanes 1, 2 and 3). In all cases, the higher molecular mass bands consistent with coupling between MDH and Trx disappeared and were resolved into two lower molecular mass bands corresponding respectively to the molecular mass of Trx and MDH subunit. These observations provide evidence that MDHs and C39S Trx form a mixed disulphide via Cys-207 of MDH and Cys-36 of Trx.

The relative quantities of each complex were calculated (Table 2B). The amount of complex formed was rather discrete with Δ-15 MDH. It was significantly increased using NDM and ΔN mutants. The difference between these two mutants was very slight, providing evidence that accessibility of Cys-207 is almost identical in both mutants, despite the fact that NDM MDH is a dimer and ΔN a monomer [1]. These results show that the opening of the Cys-24–Cys-29 bridge improves the accessibility to Cys-207, a result consistent with previous gel-filtration experiments which suggested that the reduction of the N-terminal disulphide weakened the in-

teraction between subunits [1]. They also confirmed the activation model previously proposed in which the reduction of the Cys-24–Cys-29 disulphide bridge precedes the formation and the reduction of the Cys-24–Cys-207 disulphide bridge.

4. Discussion

Our results show unambiguously that the internal cysteine of NADP-MDH able to form mixed disulphides with Trx is Cys-207: no mixed disulphide is formed when this Cys is absent. While this finding indicates that this cysteine is sufficiently reactive to form disulphide bonds, the simplified MDH model constituted by ΔN MDH does not take into account the accessibility problems of the very compact oxidised Δ-15 MDH. However, a small quantity of complex can be formed with the Δ-15 MDH, suggesting that this cysteine can be reached by Trx to some extent. This observation is consistent with our current activation model where the N-terminal disulphide present in the oxidised MDH should be the Cys-24–Cys-29 disulphide, leaving the Cys-207 in the reduced form. On the basis of size-exclusion chromatography [1], it has been proposed that the reduction of this disulphide loosens the interaction between subunits. This observation is further substantiated by the finding that the NDM mutant, which mimics the reduction of the N-terminal disulphide, is able to form higher amounts of complex than the Δ-15 enzyme. In fact, the amount of complex is identical to that observed with the deletion mutant (Table 2). The present findings strengthen the hypothesis of the role of Cys-207 in the activation process.

Trx *h* from *Chlamydomonas* proved to be very convenient in this study, having no additional cysteines besides those belonging to the active site. The reactivity of its single cysteine mutants in MDH activation tests is consistent with the reactivity data available for other Trx in types [17,18] and the pK_as of the Trx *h* cysteines (7.0 for Cys-36, 9.5 for Cys-39) determined recently [19]. The primary nucleophile of this Trx is the most N-terminal cysteine of the active site, as in all the other Trxs studied so far. However, in contrast to the report of Brandes et al. [6], no significant difference was found in the mixed disulphide forming ability of these mutants. This situation is not unusual: a similar lack of specificity of single-cysteine mutants has been reported by Wang et al. [5] for mixed disulphide formation between Trx and Trx reductase from *E. coli*. Then mixed disulphide formation between Trx and its target using chemical oxidants seems to be less specific than activity measurements. It seems to reflect more the accessibility than the reactivity of the cysteines.

The approach used in the present study to determine the accessibility of Cys-207 might prove useful for the determination of the cysteines of the N- and C-terminal disulphides of oxidised NADP-MDH which interact with Trx to form a mixed disulphide as the first step in the reduction process. Single-cysteine mutants of each of the disulphides will be necessary for this purpose.

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