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An Internal Cysteine Is Involved in the Thioredoxin-dependent Activation of Sorghum Leaf NADP-malate Dehydrogenase

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Eric Ruelland, Martine Lemaire-Chamley, Pierre Le Maréchal, Emmanuelle Issakidis-Bourguet, Nathalie Djukeic, and Myroslawa Miginiac-Maslow

From the Institut de Biotechnologie des Plantes, ERS 569 CNRS, Bâtiment 630, Université de Paris-Sud, 91405 Orsay Cedex, France

The chloroplastic NADP-malate dehydrogenase is activated by thiol/disulfide interchange with reduced thioredoxins. Previous experiments showed that four cysteines located in specific N- and carboxyl-terminal extensions were implicated in this process, leading to a model where no internal cysteine was involved in activation. In the present study, the role of the conserved four internal cysteines was investigated. Surprisingly, the mutation of cysteine 207 into alanine yielded a protein with accelerated activation time course, whereas the mutations of the three other internal cysteines into alanines yielded proteins with unchanged activation kinetics. These results suggested that cysteine 207 might be linked in a disulfide bridge with one of the four external cysteines, most probably with one of the two amino-terminal cysteines whose mutation similarly accelerates the activation rate. To investigate this possibility, mutant malate dehydrogenases (MDHs) where a single amino-terminal cysteine was mutated in combination with the mutation of both carboxyl-terminal cysteines were produced and purified. The C29S/C365A/C377A mutant MDH still needed activation by reduced thioredoxin, while the C24S/C365A/C377A mutant MDH exhibited a thioredoxin-insensitive spontaneous activity, leading to the hypothesis that a Cys24/Cys207 disulfide bridge might be formed during the activation process. Indeed, an NADP-MDH where the cysteines 29, 207, 365, and 377 are mutated yielded a permanently active enzyme very similar to the previously created permanently active C24S/C29S/C365A/C377A mutant. A two-step activation model involving a thioredoxin-mediated disulfide isomerization at the amino terminus is proposed.

NADP-dependent malate dehydrogenase (NADP-MDH)\(^{1,2}\) (EC 1.1.1.82) catalyzes the reduction of oxaloacetate into malate in higher plants. In C\(_4\) plants, such as sorghum or maize, it is located in the chloroplasts of mesophyll cells where it participates in the exportation of reducing equivalents needed for the photosynthetic electron transfer and the ferredoxin/thioredoxin system and corresponds to the reduction of disulfides present in the inactive form (3). By thiol derivatization before and after activation and site-directed mutagenesis, the disulfide bridges reduced by thioredoxins have been identified (4, 5). To reach full activity, two disulfide bridges must be reduced: an amino-terminal one (cysteines 24 and 29) and a carboxyl-terminal one (cysteines 365 and 377). When these four cysteines are mutated, the mutant protein is permanently active. The two regulatory disulfide bridges belong to two sequence extensions characteristic of the NADP-dependent isoform and absent from the NAD-dependent isoforms (6). It has been proposed that the carboxyl-terminal extension shielded the access to the active site in the oxidized form and moved upon reduction (7), whereas the reduction of the amino-terminal bridge triggered a slow conformational change shaping the active site in a high activity conformation (5).

The four regulatory cysteines have no catalytic role since their replacement by alanines does not impair the catalytic activity. Nevertheless, it has been shown that the NADP-MDH activity was sensitive to thiol reagents, such as iodoacetate or iodoacetamide. Moreover, the permanently active C24A/C29A/C365A/C377A mutant protein is sensitive to diamide, a reagent known to oxidize vicinal thiols into disulfides (5). This observation suggested that an internal cysteine might be important for catalytic activity and also raised the question of the possible existence of an internal disulfide bridge. The NADP-MDH contains eight cysteines, at strictly conserved positions for all the NADP-isoforms; these conserved cysteines are not found in the NADP-dependent isoforms (6). Four of those cysteines, belonging to sequence extensions, have been shown to play a role in the activation process and are not involved in catalytic activity. Concerning the four internal cysteines (cysteines 175, 182, 207, and 328), Cys\(^{175}\), located at the active site, has been studied earlier and shown to have no catalytic function (8). In the present study, we examined the possible role of the three other internal cysteines in the activity of the enzyme. Quite unexpectedly, one of them, i.e. Cys\(^{207}\), appeared to have a role in the reductive activation of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases, DNA modification enzymes, T4 DNA ligase, and T4 DNA polymerase were from Appligene. DEAE-Sepacel and Matrex Red A chromatographic supports were respectively from Pharmacia and Grace-Amicon. Chemicals (from Sigma, Boehringer, or Prolabo) were of analytical grade. Oligonucleotides were purchased from Eurogentec. Radiolabels were from Amersham Corp.

Escherichia coli strain XL1 blue (CLONTECH) was used to produce high yields of plasmids and M13 single-stranded DNA. All the other strains and vectors were the same as described in Ref. 9. **E. coli** strain

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\(^{‡}\) To whom correspondence should be addressed. Tel.: 33-1-69-33-63-39; Fax: 33-1-69-33-64-23; E-mail: miginiac@ibp.u-psud.fr.

\(^{1}\) The abbreviations used are: NADP-MDH, NADP-malate dehydrogenase; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; OAA, oxaloacetate; WT, wild-type.
Oligonucleotides used to introduce site-directed mutations into the mdh cDNA

| Introduced modification | Mutagenic primer sequence       |
|------------------------|--------------------------------|
| C175A                  | pGAAATCCGCCGAGCACTA            |
| C182A                  | pCAGCTAGGCCGAGCACTA           |
| C207A                  | pGAGACAAAGCAGCACGTA           |
| C207S                  | pGAGACAAAGCACGCGTGA           |
| C328A                  | pCAGACCCGAGCACTA             |

The bases underlined were introduced to replace the cysteins (written in bold) initially encoding a cysteine with codons coding for a serine or an alanine.

The enzymes were activated at 25 °C, in 100 mM Tris-HCl buffer, pH 7.9, by 20 μM E. coli thioredoxin, reduced either chemically with 10 mM DTT or photochemically in a reconstituted light activation system (14). Recombinant E. coli thioredoxin was purified as in Ref. 15. NADP-MDH activity was measured in aliquots, at 30 °C, by following the decrease in absorbance at 340 nm, in a standard assay mixture (1 ml) containing 100 mM Tris-HCl, pH 7.9, 780 μM oxaloacetate, and 140 μM NADPH. The $K_m$ values for NADPH and oxaloacetate were determined on preactivated enzymes, unless otherwise indicated, by varying the concentrations of each substrate, the other being kept constant. $S_0$ for thioredoxin were determined by measuring the initial rates of NADP-MDH activation in the presence of different concentrations of thioredoxin.

**DEPC Treatment**—The sensitivity of the C207A and C207A/C365A/C377A mutant MDHs to inhibition by DEPC was tested by adding the reagent at a 1 mM concentration either before or after activation of the enzymes in the same conditions as described in Refs. 7 and 16. Substrate protection experiments were performed on the C207A/C365A/C377A mutant by adding 1 mM NADPH before the DEPC treatment. After the treatment, the enzyme was extensively dialyzed and submitted to activation by DTT-reduced thioredoxin.

**RESULTS**

Production and Study of the NADP-MDHs Mutated on Internal Cysteines—As mentioned in the introduction, the NADP-MDH mutated on Cys175 had been studied earlier (7). To get a complete picture of the roles of cysteines in NADP-MDH, the three other internal cysteines, e.g., Cys182, Cys207, and Cys328, were individually mutated into alanines. After production in E. coli and purification to homogeneity, the three mutant proteins were found to be still active after activation by reduced thioredoxins. It thus appeared clearly that none of the internal cysteines was necessary for the catalytic process. No spontaneous activity could be measured prior to activation. Activation kinetics were followed by measuring NADP-MDH activity as a function of time after addition of thioredoxins reduced either chemically by DTT (Fig. 2) or photochemically in a reconstituted light activation system (data not shown). The activation time courses of the C182A and C328A mutant proteins, as well as the half-saturation concentrations for thioredoxin ($S_0$), were similar to those of the wild-type enzyme and of the C175A mutant studied previously (7). In contrast, the C207A mutant protein exhibited an accelerated activation time course and a 2 to 3 times lower $S_0$ for thioredoxin (Table II).

The kinetic parameters of the mutant proteins were also determined after full activation (see Table II). No significant differences for the $K_m$ OAA could be detected between the proteins mutated on Cys182, Cys207, and C328 and the WT enzyme. The $K_m$ NADPH of the C328A NADP-MDH was close to that of the WT enzyme. The $K_m$ NADPH of the C182A and C207A mutants was about 4 times higher than that of the WT enzyme (Table II).

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2 times). Thus the presence of a cysteine at position 328 seemed to be favorable for the catalytic activity even though it was not directly implicated in the catalytic process. The higher \( K_m \) NADPH of the C182A NADP-MDH suggested a possible role of Cys\(^{182} \) in the coenzyme binding, in accordance with its predicted position, close to the nicotinamide ring of the cofactor (5).

The overall effects of these individual mutations on the catalytic efficiencies of the mutated proteins \((k_{cat}/K_m)\) were rather limited, increased \( k_{cat} \) being compensated by increased \( K_m \) for NADPH. In contrast, the acceleration of the activation rate upon replacement of Cys\(^{207} \) by an alanine was rather unexpected and suggested that this cysteine could be involved in the activation process.

To strengthen this hypothesis, this residue was also replaced by a serine. The C207S mutant protein exhibited the same kinetic parameters as the C207A mutant protein, that is a \( K_m \) OAA close to that of the wild-type enzyme, and a \( K_m \) NADPH about 3 times higher (Table II). Concerning the activation process, the C207S mutant protein exhibited an accelerated activation time course and a lower activation rate. In contrast, the C207A mutant protein exhibited the same behavior as the C207S. Concerning the activation process, the C207A mutant protein exhibited an accelerated activation rate (9) and loosened the interaction between subunits (17) but did not give access to the active site. The kinetic effects of mutation of Cys\(^{207} \) into alanine or serine described above strongly suggested that this residue was involved in the activation process but did not identify the step in which this cysteine was implicated.

The role of the carboxyl-terminal bridge had been suggested first by the existence of a slight spontaneous activity in the mutants where the carboxyl-terminal disulfide had been removed. Supporting evidence for this role came from experiments using DEPC, a histidine-derivatizing reagent, which has been shown to constitute a good probe of the accessibility of the active site (16): it inhibits the enzyme activity only when the carboxyl-terminal disulfide bridge is open by reduction or suppressed by site-directed mutagenesis. To investigate a possible role of Cys\(^{207} \) in the accessibility of the active site, the effect of DEPC was tested before and after the activation of the C207A mutant MDH. The activated mutant was inhibited by DEPC to the same extent as the activated WT enzyme (data not shown), but no inhibition occurred when the reagent was applied prior to activation, i.e., the mutant enzyme could be fully activated by reduced thioredoxin after removal of DEPC (Fig. 3). In contrast, when the C207A mutation was combined with the double mutation C365A/C377A which eliminates the carboxyl-terminal disulfide, the unactivated enzyme was inhibited by DEPC and could not be activated further (Fig. 3). As in the case of the double carboxyl-terminal mutant, NADPH provided a protection against inhibition (data not shown). Clearly, the mutation of Cys\(^{207} \) alone did not give access to the active site. Interestingly, the combined triple mutant exhibited a higher spontaneous activity than the corresponding double carboxyl-terminal mutant: about 20% of the activity of the activated enzyme (Fig. 3) versus about 5% for the latter (5). Thus, a \( K_m \) for NADPH and a \( K_m \) for oxaloacetate could be measured for the non-activated triple mutant enzyme (Table III). The \( K_m \) for oxaloacetate of this enzyme prior to activation was found to be 220 \( \mu \)M, a value falling between the \( K_m \) of the fully activated C207A/C365A/C377A triple mutant or WT enzymes (approximately 38 \( \mu \)M, Tables II and III) and the \( K_m \) of the non-activated C365A/C377A NADP-MDH (1100 \( \mu \)M, Ref. 5). In other words, in the enzyme mutated on Cys\(^{207} \), the conformation of

![Figure 2: Activation kinetics of NADP-MDH bearing single mutations on internal cysteines](image-url)

**FIG. 2. Activation kinetics of NADP-MDH bearing single mutations on internal cysteines.** The wild-type and mutant MDHs were activated by 20 \( \mu \)M thioredoxins reduced by 10 \( \mu \)M DTT. The results are expressed as percentage of maximum activity. The activation rates of the proteins mutated on Cys\(^{207} \) are similar to those of the newly studied Cys\(^{207} \), have no direct catalytic role in NADPH-MDH, but that Cys\(^{207} \) seemed to be implicated in the activation process.

**Involvement of Cysteine 207 in the Activation by Thioredoxins**—In previous studies (5, 7), we have shown that the removal of the carboxyl-terminal disulfide of NADP-MDH by mutation of cysteines 365 and/or 377 opened the access to the active site but did not accelerate the slow activation rate of the enzyme which was linked to the presence of the amino-terminal disulfide. In contrast, mutation of amino-terminal cysteines (Cys\(^{24} \) and/or Cys\(^{29} \)) accelerated the activation rate (9) and loosened the interaction between subunits (17) but did not give access to the active site. The kinetic effects of mutation of Cys\(^{207} \) into alanine or serine described above strongly suggested that this residue was involved in the activation process but did not identify the step in which this cysteine was implicated.

| TABLE II | Kinetic parameters of the wild-type and mutant NADP-MDHs |
|----------|----------------------------------------------------------|
| kin | \( K_m \) NADPH | \( K_m \) Oxaloacetate | \( k_{cat}/K_m \) NADPH | \( k_{cat}/K_m \) Oxaloacetate | \( S_{1/2} \) thioredoxin* |
|---------|-----------------|-------------------------|--------------------------|-----------------------------|--------------------------|
| Wild-type | 630 | 40 (± 8) | 37 (± 5) | 16 | 17 | 10.5 (± 0.5) |
| C182A | 3000 | 111 (± 13) | 65 (± 7) | 27 | 46 | 12.0 (± 0.5) |
| C207A | 1532 | 123 (± 34) | 72 (± 33) | 14 | 21 | 3.9 (± 0.2) |
| C207S | 1073 | 123 (± 34) | 123 (± 60) | 9 | 9 | 4.2 (± 0.1) |
| C328A | 318 | 20 (± 3) | 26 (± 8) | 16 | 33 | 13.8 (± 0.4) |

*The \( S_{1/2} \) thioredoxin for the C24S, C29S, and C24S/C29S mutants was 3 \( \mu \)M (Ref. 9).
the oxaloacetate binding site was intermediary between the low affinity conformation of the unactivated wild-type enzyme and the high affinity conformation of the fully activated wild-type enzyme. This was an additional indication that Cys207 is implicated in the activation process. More specifically, this result suggested that this cysteine is involved in the conformational change of the active site proposed to occur upon the reduction of the amino-terminal bridge. Another feature suggesting that the Cys207 mutation exhibited characteristics similar to those of the amino-terminal Cys mutations rather than the carboxyl-terminal Cys mutations is the sensitivity of the activation of this mutant to inhibition by NADP (data not shown), which is a well known property of the WT enzyme and amino-terminal mutants and which is suppressed by mutation of the carboxyl-terminal cysteines (5). The question can be raised of whether, as is the case for the amino-terminal cysteine mutations, the mutation of Cys207 loosened the interaction between subunits. To answer this question, size exclusion chromatography experiments were run on the unactivated C207A mutant under varying ionic strength conditions. However, the oxidized protein behaved much like the WT protein, i.e., could not be dissociated into monomers under these conditions (data not shown).

**Function of Cysteine 207 in the Activation by Thioredoxin**—In a protein, the role of a cysteine residue can be related either to its hydrogen bonding capacity or to its hydrophobicity or to its ability to form a disulfide bridge. As the hydrophobic properties of cysteine are shared by alanine, and its hydrogen-bonding properties are similar to those of a serine, the identical results obtained with the mutations C207A and C207S are strongly in favor of the hypothesis that Cys207 reacts with another cysteine to form a disulfide bridge. In this case, a partner cysteine should be found, the mutation of which should yield the same modifications in the activation kinetics as the mutation of Cys207. None of the mutations of the other internal cysteines (cysteines 175, 182, and 328) altered the activation kinetics. Furthermore, combined mutations of each of these cysteines with Cys207 yielded proteins showing properties identical to those of the single Cys207 mutant (data not shown). Thus the formation of a disulfide bridge between any of these cysteines and Cys207 can be ruled out. Among the four regulatory cysteines located in extensions, single or double mutation of Cys365 and Cys377 did not yield accelerated activation time courses (5). Furthermore, as described above, the single mutation of Cys207 did not open the access to the active site and did not relieve the inhibition of activation by NADP. Thus it is highly unlikely that the partner cysteine of Cys207 would be one of the carboxyl-terminal cysteines. In contrast, the effects of mutation of Cys207 shared characteristics common with the mutation of the amino-terminal cysteines 24 and/or 29: accelerated activation time course and decreased $K_{1/2}$ for thioredoxin (9). This suggested that the partner cysteine of Cys207 in a disulfide bridge might be one of the two amino-terminal cysteines.

From previous studies, it had been concluded that the activation of NADP-MDH required the reduction of only two disulfides per monomer (5). This conclusion was based on the observation that both single or double amino-terminal mutations similarly accelerated the activation kinetics and that the combined mutation of the two most amino-terminal cysteines with either or both of the two most carboxyl-terminal cysteines yielded permanently active proteins. However no attempt to differentiate between possible different roles of Cys24 and Cys29 in proteins where the carboxyl-terminal bridge had been removed by mutation had been made. To fill this gap, proteins in which a single mutation of one of the two amino-terminal cysteines was combined with the mutation of the two carboxyl-terminal cysteines were produced. The biochemical properties of the C24S/C365A/C377A NADP-MDH resembled those of the previously created C24S/C29S/C365A/C377A quadruple mutant. It was permanently active, and its activity could be followed throughout the purification procedure without preacti-

![Image](65x289 to 290x474)

**FIG. 3.** Test of the accessibility of the active site of the C207A mutant NADP-MDH either singly or combined with the mutation of the two carboxyl-terminal cysteines. The unactivated enzymes were treated with 1 mM DEPC for 5 min. Then the reagent was eliminated by dialysis, the activation medium (DTT + thioredoxin) was added, and the activity was measured on aliquots as a function of time. C207A/2C, C207A/C365A/C377A.

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**TABLE III**

**Kinetic parameters of mutant NADP-MDHs**

The $K_m$ for substrates of the oxidized proteins were determined either by varying NADPH from 2.8 to 280 $\mu$M at saturating concentrations (3.75 mM) of oxaloacetate or oxaloacetate from 7.5 $\mu$M to 7.5 mM at saturating concentration (140 $\mu$M) of NADPH. The $K_m$ for substrates of the reduced proteins were determined on proteins fully activated by preincubation by 20 $\mu$M thioredoxin and 10 mM DTT. The NADPH ranged from 2.8 to 280 $\mu$M at a saturating concentration (780 $\mu$M) of oxaloacetate, and oxaloacetate ranged from 7.5 $\mu$M to 1.5 mM at a saturating concentration (140 $\mu$M) of NADPH. The $S_{1/2}$ thioredoxin were determined by varying the thioredoxin concentration in the preincubation medium from 0.5 to 40 $\mu$M and measuring the activation rate of the enzyme in the presence of saturating substrate concentrations.

| Protein                  | OAA $K_m$ $\mu$M | NADPH $K_m$ $\mu$M | $S_{1/2}$ thioredoxin $\mu$M |
|--------------------------|-------------------|---------------------|-------------------------------|
| C24S/C365A/C377A         | 57 ($\pm$ 15)     | 128 ($\pm$ 39)      |                               |
| C29S/C207A/C365A/C377A   | 79 ($\pm$ 5)      | 100 ($\pm$ 32)      |                               |

| Protein                  | Oxidized $S_{1/2}$ | Reduced $S_{1/2}$ |
|--------------------------|--------------------|-------------------|
| C207A/C365A/C377A        | 220 ($\pm$ 72)     | 38 ($\pm$ 5)      |
| C29S/C365A/C377A         | 477 ($\pm$ 66)     | 82 ($\pm$ 22)     |

The $S_{1/2}$ thioredoxin of the C365A/C377A mutant was 13 $\mu$M (Ref. 5).

The $K_m$, OAA of the oxidized C365A/C377A mutant was 1100 $\mu$M (Ref. 5).
Internal Cysteine of NADP-malate Dehydrogenase

Fig. 4. Activation of the triple mutant MDHs by reduced thioredoxins or by chemical reductants. C24/2C, C24S/C365A/C377A; C29/2C, C29S/C365A/C377A; C207/2C, C207A/C365A/C377A. The C29/2C mutant was treated either with 20 μM thioredoxin + 10 mM DTT or with 10 mM DTT or with 100 mM β-mercaptoethanol. The C29/2C mutant was treated either with 20 μM thioredoxins + 10 mM DTT or with 10 mM DTT. The C24/2C mutant was treated with 1 mM DTT. After addition of the reductant, the activity was assayed on aliquots as a function of time. The results are expressed in percent of maximum activity. The activity was assayed with 780 μM OAA (a concentration which is not saturating for the unactivated triple mutants).

Fig. 5. Light-dependent activation of combined Cys mutant NADP-MDHs in a complete reconstituted light activation system. The activity was assayed on aliquots as a function of time after turning on the light. Single or double amino-terminal mutations were introduced into a mutant MDH where the two most carboxyl-terminal cysteines had been substituted by site-directed mutagenesis. C24/2C, C24S/C365A/C377A; C29/2C, C29S/C365A/C377A; C207/2C, C207A/C365A/C377A; C29/C207/2C, C29S/C207A/C365A/C377A; C29/207/2C, C29S/C207A/C365A/C377A. The results are expressed as percentage of maximum activity.

Form a disulfide bridge with Cys29, as shown previously, or alternately with Cys207. If this interpretation is valid, a quadruple mutant combining the mutation of Cys29 and Cys207 should yield a permanently active protein. This was indeed the case (Fig. 5). The C29S/C207A/C365A/C377A NADP-MDH was fully active, and its activity could not be enhanced by reduced thioredoxin. Its kinetic properties were very similar in all respects to those of the previously created C24S/C29S/C365A/C377A quadruple mutant (5) and, more generally, to those of the activated WT enzyme (Table III).

DISCUSSION

The present work where the roles of Cys182, Cys207, and Cys328 of NADP-MDH were examined allows us to draw a general picture of the roles of the eight strictly conserved cysteines of NADP-MDH. It rules out the direct involvement of any cysteine in the catalytic process. It confirms the previously proposed hypothesis that the four cysteines belonging to sequence extensions participate in the activation of the enzyme. Among the internal cysteines, Cys182 and Cys328, as well as the previously studied Cys177 (8) clearly have no role in activation. Their substitution by site-directed mutagenesis did not modify the activation kinetics. In contrast, mutation of Cys207 into either Ala or Ser yielded mutants with accelerated activation rates. This suggested that the role of Cys207 was neither related to its hydrophobicity nor to its hydrogen-bonding capacity but specifically to its ability to form disulfide bridges. This conclusion was rather unexpected, as none of the three other internal cysteines induced similar modifications upon mutation, and hence none of them could be a partner of Cys207. On the other hand, the four external cysteines had already been shown to be paired: Cys24 with Cys29 and Cys365 with Cys377. A closer analysis of the properties of the Cys207 single mutant proteins showed that they shared a number of characteristics with the proteins mutated on the Cys of the amino-terminal extension. Their activation rates were accelerated, and their half-saturation concentrations for thioredoxin were lowered to the same extent (9). In contrast, they did not show any of the characteristics of the enzymes mutated at the two most carboxyl-terminal cysteines, i.e., slight spontaneous activity, slow activation kinetics, accessibility of the active site histidine to DEPC in the...
unactivated form, and lack of inhibition of activation by NADP (5). In an attempt to identify unequivocally, among the three cysteines yielding similar functional consequences upon mutation, the cysteine pair implicated in activation, cumulated mutations of each of these cysteines were performed on enzymes where the carboxyl-terminal bridge was removed by mutagenesis. The functional consequences of these mutations are summarized in Fig. 6. When a permanently active, thioredoxin-insensitive enzyme was obtained, it could be concluded that no reducible disulfide remained. If the mutant enzyme was still activable by reduced thioredoxin, the position of the remaining disulfide could be identified. From Fig. 6 it is clear that a thioredoxin-reducible disulfide can be formed between Cys24 and Cys29 (the previously identified amino-terminal regulatory disulfide) and also between Cys24 and Cys207. The question of the physiological relevance of these alternate disulfides is of obvious importance. The mere fact that both of them were much more efficiently reduced by thioredoxin than by DTT suggests that both belong to a physiological process. The presence of the Cys24-Cys29 disulfide was first established on the basis of chemical derivatization and amino acid sequencing. In the unactivated enzyme, both amino-terminal cysteines were unavailable to derivatization and hence appeared as gaps in amino acid sequencing. Both became available (and visible in the primary sequence) after full activation of the enzyme (4). Based on this evidence, the amino-terminal disulfide initially present in the oxidized enzyme can be identified as the Cys24-Cys29 disulfide. The mutant protein where only this disulfide remained was activated much more efficiently by reduced thioredoxin than by DTT alone. It could be fully activated by DTT, although much more slowly. The mutant protein where only the Cys24-Cys207 bridge remained was also activated very efficiently by reduced thioredoxin but could not be activated by DTT alone under standard low ionic strength conditions. Similarly, under the same conditions, the WT protein could not be activated by DTT alone, the only difference between the mutant and WT proteins being the slower activation rate of the WT enzyme. These observations strongly suggest that the Cys24-Cys207 bridge is a thioredoxin-reducible physiologically important disulfide bridge formed during the activation process and that the faster activation of the mutant protein containing this single disulfide results from the fact that one activation step had been suppressed by mutation, namely, the reduction of the amino-terminal Cys24-Cys29 disulfide. The slow activation rate linked to the reduction of the amino-terminal disulfide bridge has been previously ascribed to a slow conformational change following reduction, the disulfide reduction per se being fast. The present mutagenesis experiments suggest that this conformational change is linked to the isomerization of amino-terminal disulfides followed by a second reduction step. It has been shown previously that the elimination of the amino-terminal disulfide by site-directed mutagenesis (9) or by proteolysis (18, 19) weakened the interaction between subunits. No such effect was observed upon mutation of Cys207. On the other hand, mutation of Cys207 or, to a lesser extent, Cys29 combined with the mutation of the two carboxyl-terminal cysteines led to a decrease in the K_m OAA of the unactivated proteins (Table III), suggesting that these residues (and especially Cys207) are involved in the conformational change of the active site toward a higher efficiency.

In an attempt to visualize the succession of events following the reduction of the Cys24-Cys29 disulfide bridge, we propose that this reduction would be followed by the formation and further reduction of a Cys24-Cys207 disulfide bridge, the formation of this second disulfide being favored by the loosening of the interaction between subunits. This proposal is supported by structural data. The core part of NADP-MDH has been modeled using the coordinates of the crystal structure of NAD-MDH (5, 20). In the model, Cys207 appears to be located at the subunit interface, in a half-buried position. A change in the interaction between subunits can bring it into a more exposed position. In this regard, it should be mentioned that Cys207 was one of the cysteines Reng et al. (21) proposed to be implicated in the activation process of pea NADP-MDH and that an amino-terminally truncated pea MDH is able to form mixed disulfides with glutathione (22).

**Summary of the results of combined cysteine mutations of NADP-MDH.** The residues replacing the cysteines are written in bold; the putative disulfide bridges are visualized by a link between cysteines.

**Fig. 6.** Model of the activation of dimeric NADP-MDH by thioredoxin. The carboxyl-terminal extensions are on the outside of each monomer, close to the opening of the active site. The amino-terminal extensions are at the dimer contact area. The coenzyme binding site is figured using the coordinates of the crystal structure of NAD-MDH (5, 20). In the oxidized state, Cys207 which is situated in the vicinity of dimer contact area is half-buried. The interaction between subunits can bring it into a more exposed position. In this regard, it should be mentioned that Cys207 was one of the cysteines Reng et al. (21) proposed to be implicated in the activation process of pea NADP-MDH and that an amino-terminally truncated pea MDH is able to form mixed disulfides with glutathione (22).
The updated functional model we propose for NADP-MDH activation (Fig. 7) accommodates all the experimental data gathered from site-directed mutagenesis experiments. It is also consistent with the existence of the “preregulatory” disulfide bridge proposed by Hatch and Agostino (23). These authors observed that a pretreatment of NADP-MDH with mercaptoethanol did not activate the enzyme but accelerated its further activation by reduced thioredoxin. Such a result can be expected if mercaptoethanol reduced the Cys24–Cys207 bridge but was unable to reduce either the Cys24–Cys207 bridge formed subsequently (Fig. 5) or the Cys365–Cys377 bridge (5, 9).

In the light of the present results, the reductive activation of NADP-MDH by thioredoxin can be viewed as a protein unfolding pathway, the activation resulting not only from reduction of disulfides but also from their transient isomerization leading to a conformational change of the active site. Until now, isomerization of disulfides was shown to be an obligate step of the protein folding pathway (24), but recently it has been demonstrated that isomerization could also occur upon an unfolding triggered by disulfide reduction (25). Correct protein folding is expected if mercaptoethanol reduced the Cys24–Cys29 bridge but ethanol did not activate the enzyme but accelerated its further activation by reduced thioredoxin. Such a result can be expected if mercaptoethanol reduced the Cys24–Cys207 bridge formed subsequently (Fig. 5) or the Cys365–Cys377 bridge (5, 9).

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