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

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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 Cys<sup>24</sup>-Cys<sup>207</sup> 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.

are permanently active, the NADP-dependent isoform is totally inactive in the dark and activated in the light (2). It is now clearly established that this activation is mediated via 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 reoxidize 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 NAD-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<sup>175</sup>, 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<sup>207</sup>, appeared to have a role in the reductive activation of the enzyme.

NADP-dependent malate dehydrogenase (NADP-MDH)<sup>1</sup> (EC 1.1.1.82) catalyzes the reduction of oxaloacetate into malate in higher plants. In C<sub>4</sub> 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 fixation of atmospheric CO<sub>2</sub> into organic molecules in bundle sheath cell chloroplasts (1). Among all the malate dehydrogenases studied so far, the NADP-dependent isoform exhibits a unique property. Whereas MDHs using NAD

## EXPERIMENTAL PROCEDURES

**Materials**—Restriction endonucleases, DNA modification enzymes, T4 DNA ligase, and T4 DNA polymerase were from Appligene. DEAE-Sephacel 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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<sup>1</sup> The abbreviations used are: NADP-MDH, NADP-malate dehydrogenase; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; OAA, oxaloacetate; WT, wild-type.

TABLE I  
Oligonucleotides used to introduce site-directed mutations into the *mdh* cDNA

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

| Introduced modification | Mutagenic primer sequence |
|-------------------------|---------------------------|
| C175A                   | pGGAAATCCCGCTAACACTA      |
| C182A                   | pGCGTTAATTGCCTTGAAAAATGC  |
| C207A                   | pGAGCAAAGGCCAGCTAG        |
| C207S                   | pGAGCAAAGAGCCAGCTAG       |
| C328A                   | pCAGCATGCCAGCCATCGAA      |

RZ1032 and M13mp19 phage (Pharmacia Biotech Inc.) were used to produce deoxyuridine-substituted M13 single-stranded DNA for site-directed mutagenesis, pUC9 was used when needed for cloning strategy, and *E. coli* strain BL21 (DE3) (10) was used for the production of mutated NADP-MDHs encoded by recombinant pET-8c vectors. Bacteria were grown at 37 °C on Luria broth medium; ampicillin at 50 µg/ml was added when the bacteria carried plasmids conferring drug resistance.

**Preparation of Mutated *mdh* cDNAs**—The cDNAs encoding the NADP-MDHs bearing the single mutations C207A, C207S, C182A, and C328A were obtained by site-directed mutagenesis using the method of Kunkel (11). The single-stranded M13 template used was M13*mdh*, a vector in which the wild-type NADP-MDH cDNA is cloned (9). The mutagenic oligonucleotides are presented in Table I. A screening of the mutants was performed by sequencing single-stranded M13 DNA obtained from a few plaque isolates. The cDNA coding for the NADP-MDH with the double mutation C175A/C207A was obtained using the same method and the same template except that for the annealing reaction a mixture of both mutagenic oligonucleotides was used (see Table I).

The cloning strategies used to combine the mutations are summarized in Fig. 1. The cDNAs bearing the mutations C24S/C365A/C377A, C29S/C365A/C377A, and C207A/C365A/C377A were obtained by exchanging the *NcoI-NheI* fragment of the C365A/C377A *mdh* cDNA cloned in pUC9 for the corresponding fragment of, respectively, C24S *mdh* cDNA, C29S *mdh* cDNA, and C207A *mdh* cDNA. To obtain the cDNA with the double mutation C207A/C328A, the *NheI-BamHI* fragment of the C207A/C365A/C377A *mdh* cDNA cloned in pUC9 was exchanged for the corresponding fragment of the C328A *mdh* cDNA. Because the *NheI-BamHI* encompasses cysteines 328, 365, and 377, this cloning strategy yields a cDNA containing only the mutations C207A and C328A. The C29S/C207A/C365A/C377A cDNA was obtained by exchanging the *ApaI-ApaI* fragment of the C207A/C365A/C377A *mdh* cDNA cloned in pUC9 for the corresponding fragment of the C29S/C365A/C377A *mdh* cDNA. The *NcoI-BamHI* fragments of all the mutated MDH cDNAs were transferred to a pET vector for production of the modified proteins.

**DNA Sequencing**—The DNA sequencing was done using the dideoxy chain termination method (12) (T7 sequencing kit, Pharmacia). For every mutated cDNA, the sequence of the whole *NcoI/BamHI* fragment in the expression vector was checked prior to recombinant protein production.

**Production and Purification of Recombinant NADP-MDHs**—The mutated NADP-MDHs were produced using the pET/BL21 production system. The experimental procedures for the production in *E. coli* and purification of the recombinant proteins were essentially as described in Ref. 9. The main steps consisted of ammonium sulfate fractionation, ion-exchange chromatography on DEAE-Sephacel, and affinity chromatography on Matrex Red A. When needed, a hydrophobic interaction step on TSK Phenyl 5PW was added. The column was equilibrated with 1.8 M ammonium sulfate in 0.1 M potassium phosphate buffer, pH 7.2. The enzyme was eluted with a linear decreasing gradient of ammonium sulfate (1.8–0 M) in the same buffer. The NADP-MDH was then dialyzed against 20 mM potassium phosphate buffer, pH 7.2, 1 mM EDTA and concentrated.

**Polycrylamide Gel Electrophoresis**—Proteins were separated by vertical SDS-PAGE following the method of Schägger and von Jagow (13). They were visualized by Coomassie Brilliant Blue staining.

**Protein Sequencing**—The amino-terminal sequence of the purified proteins was verified with an Applied Biosystems model 476A automated sequencer equipped with an on-line phenylthiohydantoin-derivative analyzer.

**Molecular Mass Determination by Size Exclusion Chromatography**—Gel filtration experiments were performed using a TSK 3000 SW col-

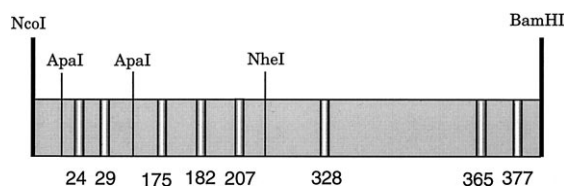


FIG. 1. Representation of the *NcoI-BamHI* fragment of the cDNA of *Sorghum vulgare* NADP-MDH. The restriction sites of *ApaI* and *NheI* are represented. The relative positions of codons encoding some cysteines are visualized; the numbers refer to the cysteines encoded.

umn (300 × 7.5 mm). After loading the protein, elution was achieved with a 30 mM Tris-HCl, pH 7.9 buffer, containing 0.15 M NaCl. Polypeptides with known molecular masses were used as standards.

**Activation and Enzyme Activity Assays**—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 photosynthetically in a reconstituted light activation system (14). Recombinant *E. coli* thioredoxin was purified as in Ref. 15. NADP-MDH activity was measured on 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.  $S_{1/2}$  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 Cys<sup>175</sup> 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. Cys<sup>182</sup>, Cys<sup>207</sup>, and Cys<sup>328</sup>, 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 photosynthetically 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 thioredoxins ( $S_{1/2}$ ), 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  $S_{1/2}$  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 cysteine 182, 207, or 328 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. When  $k_{cat}$  values of the different mutants were compared, it appeared that the replacement of cysteine 182 or 207 did not impair but increased the catalytic activity (respectively about 5- and 2-fold), whereas the replacement of Cys<sup>328</sup> by alanine resulted in a decrease of the catalytic activity (by about

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<sup>182</sup> 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<sup>207</sup> 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  $S_{1/2}$  for thioredoxin, *i.e.* characteristics similar to those of the C207A mutant (Table II and Fig. 2) and to those obtained previously for the amino-terminal mutants mutated on either or both of the most amino-terminal cysteines (9).

Based upon these experiments, it could be concluded that cysteines 182, 207, and 328, like the previously studied Cys<sup>175</sup>,

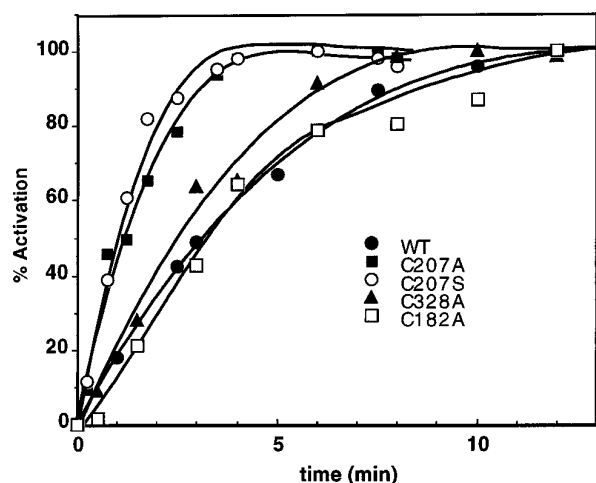


FIG. 2. Activation kinetics of NADP-MDH bearing single mutations on internal cysteines. The wild-type and mutant MDHs were activated by 20  $\mu$ M thioredoxins reduced by 10 mM DTT. The results are expressed as percentage of maximum activity. The activation rates of the proteins mutated on Cys<sup>207</sup> are similar to those of the previously studied mutants on Cys<sup>24</sup> and Cys<sup>29</sup> (Ref. 9).

have no direct catalytic role in NADPH-MDH, but that Cys<sup>207</sup> 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<sup>24</sup> and/or Cys<sup>29</sup>) 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<sup>207</sup> 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<sup>207</sup> 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<sup>207</sup> 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<sup>207</sup>, the conformation of

TABLE II  
Kinetic parameters of the wild-type and mutant NADP-MDHs

Kinetic parameters were determined at 30 °C, pH 7.9, with the purified preparations of the wild-type and mutant NADP-MDHs fully preactivated by 20  $\mu$ M thioredoxin and 10 mM DTT. The  $K_m$  for oxaloacetate was determined at a saturating concentration of NADPH (210  $\mu$ M for C207A and C207S; 140  $\mu$ M for wild-type, C182A, and C328A) with concentrations of OAA ranging from 7.5 to 1500  $\mu$ M. The  $K_m$  for NADPH was determined at a saturating concentration of oxaloacetate (780  $\mu$ M) with concentrations of NADPH ranging from 1.4 to 210  $\mu$ M. 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.

|           | $k_{cat}$ | $K_m$           |                 | $k_{cat}/K_m$                           |              | $S_{1/2}$ thioredoxin <sup>a</sup> |
|-----------|-----------|-----------------|-----------------|---|--------------|------------------------------------|
|           |           | NADPH           | Oxaloacetate    | NADPH                                   | Oxaloacetate |                                    |
|           | $s^{-1}$  | $\mu$ M         |                 | $\mu$ M <sup>-1</sup> · s <sup>-1</sup> | $\mu$ M      |                                    |
| Wild-type | 630       | 40 ( $\pm$ 8)   | 37 ( $\pm$ 5)   | 16                                      | 17           | 10.5 ( $\pm$ 0.5)                  |
| C182A     | 3000      | 111 ( $\pm$ 13) | 65 ( $\pm$ 7)   | 27                                      | 46           | 12.0 ( $\pm$ 0.5)                  |
| C207A     | 1532      | 123 ( $\pm$ 34) | 72 ( $\pm$ 33)  | 14                                      | 21           | 3.9 ( $\pm$ 0.2)                   |
| C207S     | 1073      | 123 ( $\pm$ 34) | 123 ( $\pm$ 60) | 9                                       | 9            | 4.2 ( $\pm$ 0.1)                   |
| C328A     | 318       | 20 ( $\pm$ 3)   | 26 ( $\pm$ 8)   | 16                                      | 33           | 13.8 ( $\pm$ 0.4)                  |

<sup>a</sup> 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 Cys<sup>207</sup> 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 Cys<sup>207</sup> 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 Cys<sup>207</sup> 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).

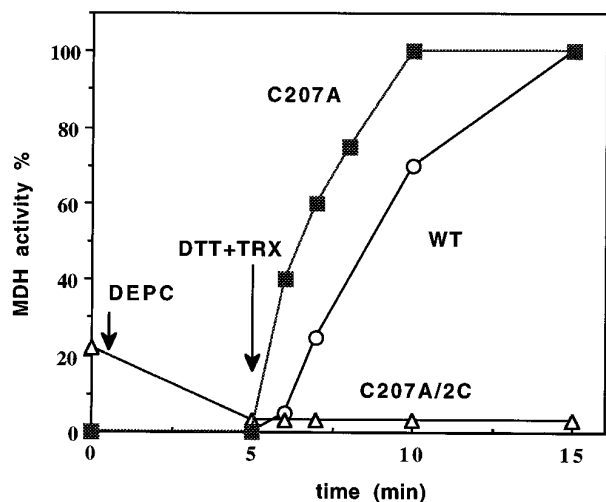


FIG. 3. Test of the accessibility of the active site of the C207A mutant NADP-MDH either singly or combined with the mutation of the two most carboxyl-terminal cysteines. The unactivated enzymes were treated with 1 mM DEPC for 5 min. Then the reagent was eliminated by dialysis, the activation medium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

*Function of Cysteine 207 in the Activation by Thioredoxins*—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 Cys<sup>207</sup> 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 Cys<sup>207</sup>. 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 Cys<sup>207</sup> yielded proteins showing properties identical to those of the single Cys<sup>207</sup> mutant (data not shown). Thus the formation of a disulfide bridge between any of these cysteines and Cys<sup>207</sup> can be ruled out. Among the four regulatory cysteines located in extensions, single or double mutation of Cys<sup>365</sup> and Cys<sup>377</sup> did not yield accelerated activation time courses (5). Furthermore, as described above, the single mutation of Cys<sup>207</sup> 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 Cys<sup>207</sup> would be one of the carboxyl-terminal cysteines. In contrast, the effects of mutation of Cys<sup>207</sup> shared characteristics common with the mutation of the amino-terminal cysteines 24 and/or 29: accelerated activation time course and decreased  $S_{1/2}$  for thioredoxin (9). This suggested that the partner cysteine of Cys<sup>207</sup> 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 Cys<sup>24</sup> and Cys<sup>29</sup> 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-

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

| Protein                | $K_m$                 |                 | $S_{1/2}$ thioredoxin <sup>a</sup> |                 |                  |
|------------------------|-----------------------|-----------------|------------------------------------|-----------------|------------------|
|                        | OAA                   | NADPH           | Oxidized                           | Reduced         |                  |
|                        | $\mu\text{M}$         | $\mu\text{M}$   | $\mu\text{M}$                      | $\mu\text{M}$   |                  |
| C24S/C365A/C377A       | 57 ( $\pm$ 15)        | 128 ( $\pm$ 39) |                                    |                 |                  |
| C29S/C207A/C365A/C377A | 79 ( $\pm$ 5)         | 100 ( $\pm$ 32) |                                    |                 |                  |
|                        | Oxidized <sup>b</sup> | Reduced         | Oxidized                           | Reduced         |                  |
| C207A/C365A/C377A      | 220 ( $\pm$ 72)       | 38 ( $\pm$ 5)   | 33 ( $\pm$ 7)                      | 52 ( $\pm$ 7)   | 4.0 ( $\pm$ 0.5) |
| C29S/C365A/C377A       | 477 ( $\pm$ 66)       | 82 ( $\pm$ 22)  | 97 ( $\pm$ 6)                      | 163 ( $\pm$ 52) | 3.8 ( $\pm$ 0.2) |

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

<sup>b</sup> The  $K_m$  OAA of the oxidized C365A/C377A mutant was 1100  $\mu\text{M}$  (Ref. 5).

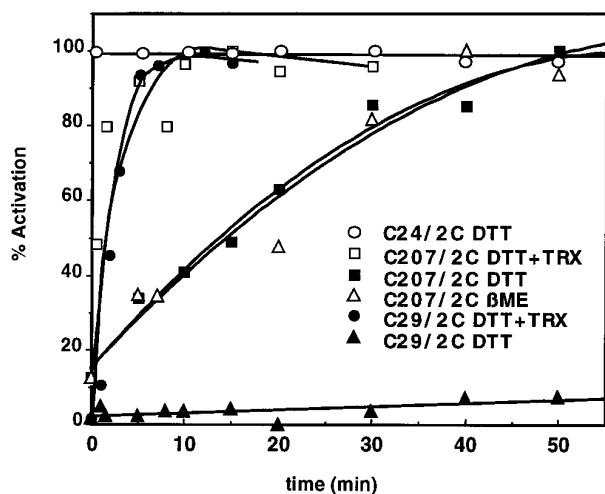


FIG. 4. Activation of the triple mutant MDHs by reduced thioredoxins or by chemical reductants. C24/2C, C24S/C365A/C377A; C29/2C, C29S/C365A/C377A; C207/2C, C207A/C365A/C377A. The C207/2C mutant was treated either with 20  $\mu$ M thioredoxin + 10 mM DTT or with 10 mM DTT or with 100 mM  $\beta$ -mercaptoethanol. The C29/2C mutant was treated either with 20  $\mu$ M 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  $\mu$ M OAA (a concentration which is not saturating for the unactivated triple mutants).

vation (data not shown). However, as it was also rather unstable and susceptible to oxidation, maximal activity of the pure protein could be obtained only if 1 mM DTT were included in the reaction medium (Fig. 4). In contrast, the C29S/C365A/C377A NADP-MDH still required activation by reduced thioredoxin to reach full activity (Fig. 4). Its activation rate was very similar to the activation rate of the C207A/C365A/C377A mutant, but its spontaneous activity was very similar to that of the C365A/C377A NADP-MDH, *i.e.* approximately 5% of the activity of the fully activated protein. The  $K_m$  OAA of the unactivated enzyme (477  $\mu$ M) was higher than that of the fully activated enzyme (66  $\mu$ M, Table III) but somewhat lower than that of the non-activated C365A/C377A NADP-MDH (1100  $\mu$ M, Ref. 5). Clearly, this mutant shared some similarities with the C207A/C365A/C377A mutant but also exhibited some notable differences. In particular, despite the fact that the thioredoxin-dependent activation of both mutants proceeded at similar rates, the C207A/C365A/C377A mutant could be fully activated either by DTT or by mercaptoethanol alone, although much more slowly, whereas the C29S/C365A/C377A mutant was strictly dependent on reduced thioredoxin for activation (Fig. 4). The thioredoxin dependence of the activation can be unambiguously established by using a reconstituted light activation system in which thioredoxins are reduced by the photosynthetic electron transfer. In this system both C207A/C365A/C377A and C29S/C365A/C377A triple mutants were activated at higher rates than was the WT protein, without the typical lag in activation of the latter (Fig. 5). In contrast, when the C24S/C365A/C377A mutant was assayed in this system after having been inactivated by dilution, its activity could not be restored unless DTT was added either to the activation medium or to the reaction cuvette (Fig. 5).

Summarizing the results obtained with the three combined triple mutations, it can be concluded that whereas in the C24S/C365A/C377A mutant there was no thioredoxin-reducible disulfide bridge left, a thioredoxin-reducible disulfide was still present in both the C29S/C365A/C377A and the C207A/C365A/C377A mutants. This observation suggested that Cys<sup>24</sup> could

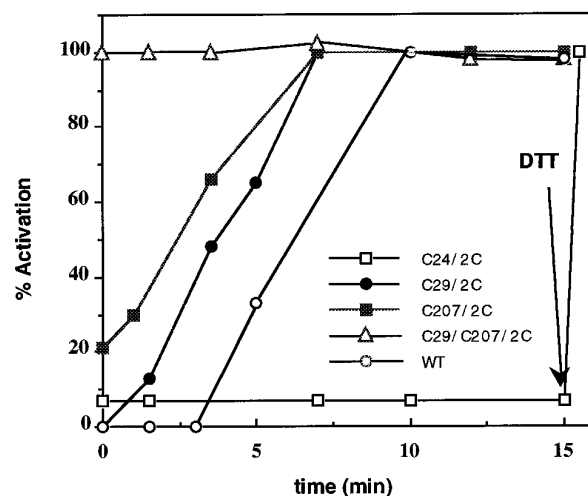


FIG. 5. Light-dependent activation of combined Cys mutant NADP-MDHs in a complete reconstituted light activation system. The activity was assayed on aliquots as a function of time after turning on the light. Single or double amino-terminal mutations were introduced into a mutant MDH where the two most 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. The results are expressed as percentage of maximum activity.

form a disulfide bridge with Cys<sup>29</sup>, as shown previously, or alternately with Cys<sup>207</sup>. If this interpretation is valid, a quadruple mutant combining the mutation of Cys<sup>29</sup> and Cys<sup>207</sup> 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 Cys<sup>182</sup>, Cys<sup>207</sup>, and Cys<sup>328</sup> 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, Cys<sup>182</sup> and Cys<sup>328</sup>, as well as the previously studied Cys<sup>175</sup> (8) clearly have no role in activation. Their substitution by site-directed mutagenesis did not modify the activation kinetics. In contrast, mutation of Cys<sup>207</sup> into either Ala or Ser yielded mutants with accelerated activation rates. This suggested that the role of Cys<sup>207</sup> 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 Cys<sup>207</sup>. On the other hand, the four external cysteines had already been shown to be paired: Cys<sup>24</sup> with Cys<sup>29</sup> and Cys<sup>365</sup> with Cys<sup>377</sup>. A closer analysis of the properties of the Cys<sup>207</sup> 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 Cys<sup>24</sup> and Cys<sup>29</sup> (the previously identified amino-terminal regulatory disulfide) and also between Cys<sup>24</sup> and Cys<sup>207</sup>. 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 Cys<sup>24</sup>-Cys<sup>29</sup> 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

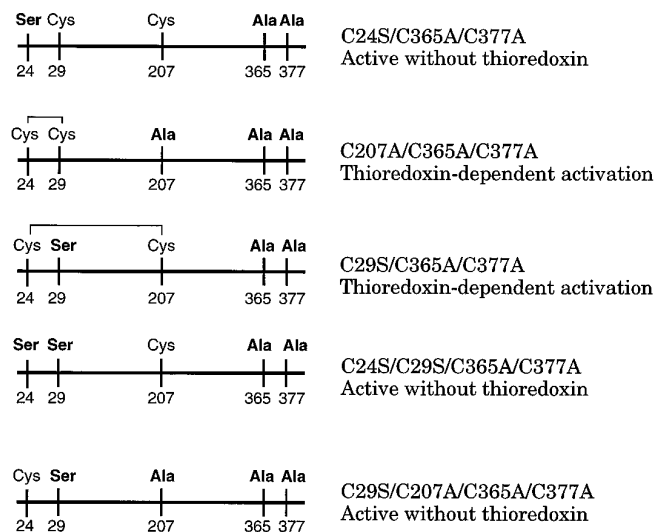


FIG. 6. Summary of the results of combined cysteine mutations of NADP-MDH. The residues replacing the cysteines are written in *bold*; the putative disulfide bridges are visualized by a link between cysteines.

present in the oxidized enzyme can be identified as the Cys<sup>24</sup>-Cys<sup>29</sup> 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 Cys<sup>24</sup>-Cys<sup>207</sup> 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 Cys<sup>24</sup>-Cys<sup>207</sup> 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 Cys<sup>24</sup>-Cys<sup>29</sup> 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 Cys<sup>207</sup>. On the other hand, mutation of Cys<sup>207</sup> or, to a lesser extent, Cys<sup>29</sup> 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 Cys<sup>207</sup>) 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 Cys<sup>24</sup>-Cys<sup>29</sup> disulfide bridge, we propose that this reduction would be followed by the formation and further reduction of a Cys<sup>24</sup>-Cys<sup>207</sup> 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, Cys<sup>207</sup> 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 Cys<sup>207</sup> 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).

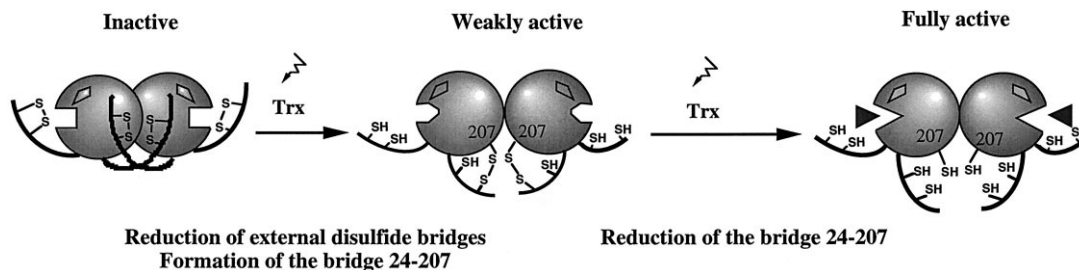


FIG. 7. Model of the activation of dimeric NADP-MDH by thioredoxins. The carboxyl-terminal extensions are on the outside of each monomer, close to the opening of the active site. The amino-terminal extensions are at the dimer contact area. The coenzyme binding site is figured by a *diamond* and oxaloacetate by a *triangle*. In the oxidized state, Cys<sup>207</sup> which is situated in the vicinity of dimer contact area is half-buried. The carboxyl-terminal extensions shield the active site in the unactivated enzyme and move upon reduction. The reduction of the amino-terminal extensions loosens the interaction between monomers, thus triggering a slow conformational change of the active site and making Cys<sup>207</sup> accessible. A Cys<sup>207</sup>-Cys<sup>24</sup> bridge is then formed. After reduction of this bridge, the conformational change of the active site is completed, yielding an active site with high affinity for OAA.

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 Cys<sup>24</sup>-Cys<sup>29</sup> bridge but was unable to reduce either the Cys<sup>24</sup>-Cys<sup>207</sup> bridge formed subsequently (Fig. 5) or the Cys<sup>365</sup>-Cys<sup>377</sup> 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 considered to be catalyzed by protein-disulfide isomerase (26), which is a strong oxidant. However, thioredoxin was also shown to possess a disulfide isomerase activity (27). The present work suggests that thioredoxin could also be involved in the isomerization of disulfides in conjunction with its disulfide reducing function. The case of NADP-MDH is the best documented among the light-activated chloroplastic enzymes in this respect. However, it is not the only known example. Recent mutagenesis experiments on the thioredoxin-regulated chloroplastic fructose-1,6-bisphosphatase showed that three cysteines were involved in the reductive activation of the enzyme (28). Thus the light regulation of some chloroplastic enzymes could be viewed as a general regulation process in which thioredoxin, in addition to its disulfide reducing activity, fulfills a disulfide isomerase function, thus ensuring a correct folding/unfolding of the light-regulated enzymes.

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