Inhibition of the Thioredoxin-dependent Activation of the NADP-malate Dehydrogenase and Cofactor Specificity*

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The chloroplastic NADP-malate dehydrogenase is activated by reduction of its N- and C-terminal disulfides by reduced thioredoxin. The activation is inhibited by NADP+, the oxidized form of the cofactor. Previous studies suggested that the C-terminal disulfide was involved in this process. Recent structural data pointed toward a possible direct interaction between the C terminus of the oxidized enzyme and the cofactor. In the present study, the relationship between the cofactor specificity for catalysis and for inhibition of activation has been investigated by changing the cofactor specificity of the enzyme by substitution of selected residues of the cofactor-binding site. An NAD-specific thiol-regulated MDH was engineered. Its activation was inhibited by NAD+ but no longer by NADP+. These results demonstrate that the oxidized cofactor is bound at the same site as the reduced cofactor and support the idea of a direct interaction between the negatively charged C-terminal end of the enzyme and the positively charged nicotinamide ring of the cofactor, in agreement with the structural data. The structural requirements for cofactor specificity are modeled and discussed.

The chloroplastic NADP-malate dehydrogenase (EC 1.1.1.82) differs from all the other malate dehydrogenases in its cofactor specificity; it uses NADPH instead of NADH. It has also an additional remarkable property; it is activated by reduction through thiol/disulfide interchange via the ferredoxin-thioredoxin system (1), whereas the other malate dehydrogenases are permanently active. This activation is specifically inhibited by the oxidized form of the cofactor (2, 3). The cofactor specificity holds for the inhibitory effect as well; NADP-MDH1 activation is inhibited by NADP+ but not by NAD+. Recent biochemical (4, 5) and structural (6, 7) studies have clarified the complex activation mechanism of the enzyme. In the oxidized form, NADP-MDH features two different disulfides per subunit: one at the N terminus and the other at the C terminus, each situated in sequence extensions typical for the thiol-regulated forms. The C-terminal bridge pulls the C-terminal extension toward the active site where it acts as an internal inhibitor, preventing substrate access to the active site. Upon reduction, the C-terminal extension is released from the active site (8). The evidence for the exact role of the N-terminal regulatory disulfide is less clear, but it has been proposed that reduction of this disulfide induces a conformational change of the protein during which substrate affinity is optimized.

These results provided some clues for the interpretation of previous observations made during studies aimed at solving the puzzling inhibitory effect of NADP+. Of particular importance were the observations that the inhibition disappeared when the C-terminal disulfide was reduced or when either of its cysteines was replaced by site-directed mutagenesis (9) and also when the negative charge of the penultimate C-terminal glutamate residue was eliminated by a substitution for a glutamine (5). The last observation suggested an interaction between the negatively charged penultimate residue and the positively charged nicotinamide ring of the oxidized cofactor but did not provide any clue about the high specificity for NADP+ versus NAD+. In particular, it remained unclear whether the specificity for inhibition and the specificity for catalysis were related or whether the inhibitory effect of NADP+ was strictly related to the particular thiol activation mechanism of the enzyme.

In an attempt to answer these questions, we have performed site-directed mutagenesis of amino acids at the cofactor-binding site of NADP-MDH to shift its cofactor specificity from NADPH toward NADH. The results indicate that the relationship between cofactor specificity for inhibition and for catalysis characteristic of the wild type enzyme is also observed in mutants with altered cofactor specificity. The results are discussed in light of recent structural data.

EXPERIMENTAL PROCEDURES

Materials—Pfu DNA polymerase, oligonucleotides, restriction endonucleases, and T4 DNA ligase were obtained from Promega, Appligene, and Life Technologies, Inc. DEAE-Sephacel, ACA 44 and Matrex red A nuclease, and T4 DNA ligase were obtained from Promega, Appligene, and Life Technologies, Inc. The mns7 ind1 Sam7 nin lacUV5-T7 gene was used for the production of wild type and mutant NADP-MDHs encoded by recombinant pET vectors (11). Bacteria were grown at 37 °C on Luria broth medium supplemented with ampicillin 50 µg when the bacteria carried pET or pUC plasmid.

Site-directed Mutagenesis of NADP-MDH cDNA and DNA Sequenc-
ing—PCR site-directed mutagenesis strategy was used to introduce the G84D, the S85I/R87Q/S88A, and the G84D/S85I/R87Q/S88A mutations into the wild type sorghum NADP-MDH cDNA sequence (GenBank accession number X53453, Ref. 12). The following oligonucleotides were used to generate the mutant proteins: NcoI and NheI restriction endonucleases. The whole cDNA bearing the NcoI–NheI fragment of wild type MDH cDNA cloned in pUC9 for the fragment obtained by PCR. The NcoI-BamHI fragment of the mutated MDH cDNA was transferred to a pET vector for production of the modified protein. The sequence of every mutated cDNA was checked after each cloning step using the method of Sanger et al. (Ref. 13; T7 sequencing kit; Amershams Pharmacia Biotech).

**Mutant Protein Production and Purification**—Procedures for expression of the mutated NADP-MDH cDNA in pET and preparation of soluble protein extracts from transformed *E. coli* were the same as described previously (10). Purification of the mutants consisted of ammonium sulfate fractionation between 35 and 60% saturation in 20 mM sodium phosphate, 1 mM EDTA, pH 7.2 buffer (PE buffer) followed by a dialysis overnight against 100 volumes of PE buffer. The fractionated protein was then loaded on a DEAE-Sepharose column (2.5 × 25 cm) washed extensively with PE buffer, and eluted with a linear gradient (2 × 250 ml) of 0–0.6 mM NaCl in PE buffer. For the S85I/R87Q/S88A and G84D mutants, the active fractions were pooled and directly loaded on a Matrix Red A column (15 × 1.5 cm). After washing with PE buffer, the enzyme was eluted from the affinity column with a linear gradient (2 × 250 ml) 0–3 mM NaCl in PE buffer. For the S85I/R87Q/S88A mutant or 0–2 mM NaCl in PE buffer. Oxidation-reduction potentials were measured on preactivated enzymes by varying the concentration of each substrate in the assay mixture.

The activation kinetics, enzyme activity assays, and kinetic parameter determinations—the enzymes were activated at 25 °C in 100 mM Tris-HCl, pH 7.9, buffer with 20 μM E. coli thioredoxin reduced by 10 mM dithiothreitol. Activity was measured on activated 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 or 140 μM NADH. The kinetic parameters were determined on preactivated enzymes by varying the concentration of each substrate in the assay mixture.

The redox potentials of the disulfides of the WT and S85I/R87Q/S88A mutated NADP-MDH have been determined as described in Ref. 15. Oxidation-reduction potentials were measured on preactivated enzymes by varying the concentration of each substrate in the assay mixture. Molecular design—To investigate the interactions between NAD* and the mutated residues in the Sorghum MDH, a hypothetical model was made. This was done using the coordinates of both the apo-form of Sorghum NADP-dependent MDH (Protein Data Bank code 7mdh) and coordinates of the holo-form of NAD-dependent *Thermus flavus* MDH (Protein Data Bank code 1bd). To find an approximate orientation of the NAD* in the Sorghum structure, the two structures were superposed using the molecular graphics program O (16). Further, the residues corresponding to the mutations in the Sorghum MDH were exchanged into the first Phe residue of the disulfide loop at the nucleotide-binding site of the *T. flavus* structure. This was also done using program O. Fig. 3 was drawn using the programs Molscript (17) and Raster3D (18).

**RESULTS**

*Choice of Mutations*—A number of studies designed to reverse the catalytic specificity of dehydrogenases toward their cofactor have already been performed. Most of them consisted of a reversal from NAD to NADP (for reviews see Refs. 19 and 20). Based on these studies and on sequence comparison between NAD- and NADP-dependent malate or lactate dehydrogenases (21, 22), we have chosen to mutate four amino acids located at the putative nucleotide-binding site of NADP-MDH. These residues are conserved among NADPH-dependent malate dehydrogenases but are replaced by a different set of conserved residues in NADP-dependent dehydrogenases (Fig. 1). The study by Nishiyama et al. (21) was particularly useful as a model for cofactor specificity because it presented data for the enzyme that is the closest in primary structure to the chloroplastic NADP-MDH, namely the NAD-MDH from *T. flavus*. This study showed that a total reversal of specificity toward NADPH required the substitution of a seven-residue loop at the nucleotide-binding site. As a rule, most of the other studies reached the conclusion that single mutations resulted in mixed cofactor specificity. Thus, in our first mutant, three amino acids were substituted at a time (mutant S85I/R87Q/S88A). Because the cofactor specificity reversal was not perfect, we added a fourth mutation (mutant G84D/S85I/R87Q/S88A) and checked also the effect of a single G84D substitution on the G84D/S85I/R87Q/S88A mutant. The redox potentials of the disulfides of the WT and S85I/R87Q/S88A mutated NADP-MDH have been determined as described in Ref. 15. Oxidation-reduction potentials were measured on preactivated enzymes by varying the concentration of each substrate in the assay mixture.
not improve the binding, thus additional purification steps were required to purify these proteins to homogeneity. This suggested that the G to D mutation is crucial for cofactor binding specificity. To further evaluate the roles of the different mutated residues in cofactor specificity, the kinetic parameters of each of the preactivated mutant proteins have been determined, using either NADH or NADPH as a cofactor (Table I).

The S85/R87Q/S88A mutation yielded a protein exhibiting almost identical $K_m$ for NADH and NADPH. This mutant also exhibited the same $k_{cat}$ values, regardless of which cofactor was used. The equalization of $K_m$ values for the two cofactors resulted from a 6-fold increase in the $K_m$ for NADPH and a 4-fold decrease in the $K_m$ for NADH. If we compare the overall catalytic efficiencies, as expressed by the ratios $k_{cat}/(K_m \text{ OAA} \times K_m \text{ cofactor})$, this mutant protein was 85 times less efficient than the wild type MDH with NADPH but 75 times more efficient than the wild type MDH with NADH. The G84D mutation also led to a protein exhibiting similar $K_m$ values for both cofactors. However, the mutation did not modify the $K_m$ for NADPH but lowered the $K_m$ for NADH 10-fold. The overall catalytic efficiency of this mutant protein was 7-fold higher with NADH than with NADPH, thus its catalytic efficiency with NADH was equal to the catalytic efficiency of the wild type protein with NADPH. A total reversal of the cofactor specificity could be obtained by combining these two mutations in the mutant G84D/S85I/R87Q/S88A. In this mutant, the $K_m$ values for cofactors and catalytic efficiencies were exactly reversed compared with the wild type NADP-MDH.

The interaction of the enzyme with its substrate, oxaloacetate, was also affected by the cofactor specificity of the mutants. Indeed, for the G84D and G84D/S85I/R87Q/S88A mutant proteins, the $K_m$ for OAA decreased 6- and 200-fold, respectively when NADH was used as cofactor instead of NADPH. For each mutant, the inhibition by excess OAA was retained, except for the G84D/S85I/R87Q/S88A mutant for which the inhibitory concentrations of OAA were shifted toward higher concentrations when NADPH was used as a cofactor (data not shown) in accordance with the high $K_m$ OAA of this mutant in these conditions.

The G84D, S85I/R87Q/S88A, and G84D/S85I/R87Q/S88A Mutant Proteins Are Alterated in Their Sensitivities to Inhibition by the Oxidized Cofactors—The activation kinetics of the mutant proteins have been determined in the presence or in the absence of the oxidized cofactor in the activation medium. Because the aliquot taken for measuring the activity is small in volume (10 µl), the cofactor is diluted 100-fold in the reaction medium and does not interfere with the activity measurement. First one can notice that the wild type, the G84D, S85I/R87Q/S88A, and G84D/S85I/R87Q/S88A mutant proteins exhibited the same activation kinetics, i.e. each of these proteins was fully activated within 10 min. Obviously, the mutations did not perturb the activation process. The results of the activation assays in the presence of either 1 mM NAD$^+$ or 1 mM NADP$^+$ are summarized in Fig. 2. Similar assays have also been done with NADH and NADPH as controls, and the reduced cofactors did not have any effect on activation kinetics of any of the proteins (data not shown). In the presence of the oxidized cofactors in the activation medium, the activation kinetics of the G84D, S85I/R87Q/S88A, and G84D/S85I/R87Q/S88A mutants differed from those of the wild type protein with respect of cofactor specificity of the inhibitory effect. Indeed, the activation of the wild type NADP-MDH was strongly inhibited by NADP$^+$ but very weakly inhibited by NAD$^+$. In contrast, the activation of each of the three mutated proteins was almost insensitive to NADP$^+$ (or even totally insensitive when the G84D mutation was introduced either alone or in combination with other amino acid substitutions) but became sensitive to NAD$^+$.

Thus, the cofactor specificity of the inhibition of the activation has been partially or completely reversed by the mutations affecting the cofactor specificity for catalysis. The inhibition results were the same whether the subsequent activity determinations were done with NADH or with NADPH (data not shown).

### DISCUSSION

**Involvement of the Residues Gly$^{84}$, Ser$^{85}$, Arg$^{87}$, and Ser$^{88}$ in Cofactor Specificity**—All the studies aimed at changing the cofactor specificity of dehydrogenases reached the conclusion that the specificity depends on several interactions and that a total reversal requires the mutation of several residues (19, 20). Compared with the results of Nishiyama et al. (21), which showed that a reversed specificity in favor of NADPH required the substitution of a seven-residue loop in T. flavus NAD-malate dehydrogenase nucleotide-binding site, our study shows that replacing the residues Ser$^{85}$, Arg$^{87}$, and Ser$^{88}$ of NADP-MDH by the corresponding residues of T. flavus NAD-MDH and residue Gly$^{84}$ by an aspartate, the most commonly found residue at this position in the NAD-dependent forms, yielded a mutant protein G84D/S85I/R87Q/S88A exhibiting a completely reversed cofactor specificity.

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**Table 1**

| Protein     | Sequence          |
|-------------|-------------------|
| Sorghum     | L K L L G S E R S F Q A L E G V |
| Maize       | L K L L G S E R S F Q A L E G V |
| Spinach     | L K L L G S E R S F Q A L E G V |
| Ice plant   | L K L L G S E R S F Q A L E G V |
| Pea         | L K L L G S E R S F Q A L E G V |
| T. flavus   | L Q L L E I P Q A M K A L E G V |
| Pig cyto    | L V L L D I T P M M G V L D G V |
| Human cyto  | L V L L D I T P M M G V L D G V |
| Pig mito    | L T L L D I A H T P G V |
| E.coli      | L S L Y D I A P V T P G V |
| Chlorobium  | I V L L D I V E G V P Q G K |
| H.marismortui| V V F V D I P D K E D D T V G Q |
The single G84D mutation resulted in a 10-fold lower $K_m$ for NADH, whereas the $K_m$ for NADPH remained unchanged. This result suggests that the aspartate residue favors the interaction with NADH but has no effect on NADPH positioning. In similar studies on some NADH-dependent dehydrogenases, namely lactate dehydrogenase (Ref. 23; mutation D52S) or NAD-MDH (Ref. 21; mutation D41G/F428A/A458S), when the homologous aspartate was mutated into a serine or a glycine, an effect on both $K_m$ and efficiency in NADH use was obtained, whereas the kinetic parameters for NADPH remained almost unchanged. In the structure of the NADH-lactate dehydrogenase complex, the aspartate was shown to hydrogen bond to the 2'- and 3'-OH of the adenosine ribose. The modeled structure of the mutated NADP-MDH (Fig. 3) suggests that a similar interaction between the introduced Asp$^{+}$ and the 2' and 3' hydroxyl of NAD$^{+}$ could also occur. Thus, the G84D mutation could provide a stabilization for NAD$^{+}$, which would not occur with NADP$^{+}$ because of steric hindrance of the phosphate group. However, studies on other NAD$^{+}$-dependent dehydrogenases such as yeast alcohol dehydrogenase I (Ref. 24; mutation D223S) or glyceraldehyde phosphate dehydrogenase (Ref. 25; mutation D32A) suggested that the negative charge of aspartate could repel the 2'-phosphate group of the ribose of NADPH. Recent structural studies on Flaveria NADP-MDH with NAD$^{+}$ bound (7) also suggest that substitution of an aspartate residue at position 84 for the glycine that normally occupies this position in the NADP-dependent dehydrogenases would introduce sufficient steric hindrance to prevent NADPH binding by the protein. This prediction was not confirmed by our biochemical results. In the G84D mutant protein the $K_m$ for NADPH remained unchanged. This suggests that NADP$^{+}$ is bound at the active site of the mutated protein in a different conformation than that shown for NAD$^{+}$ in our model (Fig. 3) or adopted by bound NADP$^{+}$ in the structure of Flaveria MDH (Protein Data Bank code 1CIV). It has been reported (19) that the conformation of bound NAD$^{+}$ is conserved in dehydrogenases, whereas bound NADP$^{+}$ can adopt a variety of conformations.

The $K_m$ for NADPH and a 4-fold decrease in the $K_m$ for NADH. We suggest that the loss of specificity toward NADPH is linked to the loss of residues able to stabilize the phosphate group, either by hydrogen bonding (serine residues) or by electrostatic interaction (arginine residue). Several studies on dehydrogenases have previously demonstrated similar effects linked to the mutation of the arginine of the cofactor-binding site. This

### Table I

**Cofactor Specificity of NADP-malate Dehydrogenase**

| Cofactor | Wild type MDH | SRS mutant | G mutant | GSRS mutant |
|----------|---------------|------------|----------|-------------|
|          | NADPH | NADH | Ratio NADPH/NADH | NADPH | NADH | Ratio NADPH/NADH | NADPH | NADH | Ratio NADPH/NADH |
| $K_m$ (Cofactor) ($\mu$M) | 50 ± 18 | 894 ± 46 | 0.056 | 332 ± 56 | 231 ± 1 | 1.43 | 106 ± 15 | 84 ± 9 | 1.26 |
| $K_m$ (OAA) ($\mu$M) | 30 ± 5 | 67 ± 6 | 0.44 | 175 ± 43 | 59 ± 7 | 2.96 | 40 ± 3 | 7 ± 31 | 0.57 |
| $k_{cat}$ (s$^{-1}$) | 600 ± 70 | 6 ± 4 | 100 | 272 ± 7 | 101 ± 3 | 2.69 | 144 ± 5 | 34 ± 11 | 1.07 |

*FIG. 2. Activation kinetics of the wild type (a), G84D/S85I/R87Q/S88A (b), G84D (c), and S85I/R87Q/S88A (d) NADP-MDH with or without the oxidized cofactor present in the activation medium.*

The activation was started by adding dithiothreitol-reduced thioredoxin to the inactive enzyme either in the presence of 1 mM NADP$^{+}$ or 1 mM NAD$^{+}$ or without any cofactor. Aliquots (10 $\mu$l) were taken out at regular time intervals and injected into a spectrophotometer cuvette containing the standard reaction medium (780 $\mu$L OAA and 140 $\mu$L NADPH for activity measurements). NADPH oxidation rate was measured as a decrease in absorbance at 340 nm. For the G84D/S85I/R87Q/S88A mutated protein (b) with totally reversed cofactor specificity, NADH was used in the reaction medium instead of NADPH. 100% activity corresponds respectively to 857 $\mu$mol (a), 104 $\mu$mol (b), 205 $\mu$mol (c), and 388 $\mu$mol (d) cofactor oxidized/mg protein/min. All of the enzymes are totally inactive without activation.
arginine is considered to be the only largely conserved structural feature in NADP-dependent dehydrogenases (19). Indeed, on the basis of structural and mutagenesis data, an equivalent arginine residue of the dual coenzyme specificity glucose-6-phosphate dehydrogenase was shown to be involved in binding NADP\(^+\) but not NAD\(^+\) via the adenosine 2'-phosphate (26). The replacement of this arginine yielded a protein with a \( K_m \) for NADPH that was increased by a factor of 750, whereas its \( K_m \) for NADH was decreased by a factor of 7. Another study on a nonhomologous dehydrogenase, glutathione reductase (27), showed that two arginine residues could be very important for NADPH specificity through the binding of the 2'-phosphate group of NADPH. The mutation of these arginines yielded a protein with a 15-fold increased \( K_m \) for NADPH and a 4-fold decreased \( K_m \) for NADH. On the other hand, several studies have shown that the serines belonging to the cofactor-binding sites of oxidoreductases were also involved in the cofactor specificity probably through hydrogen bonding to the adenosine 2'-phosphate of the cofactor. For example, a S212D mutation of cinnamyl alcohol dehydrogenase showed that this serine residue interacted directly with the adenosine 2'-phosphate. Indeed, the mutation yielded a protein with an increased \( K_m \) for NADPH but with an unchanged \( K_m \) for NADH (28). Thus, combining mutations that stabilize NADH binding with mutations that disfavor the stabilization of 2'-phosphate of NADPH yields a mutated protein with a cofactor specificity that is strikingly shifted toward the use of NADH instead of NADPH.

**Cofactor Binding Influences \( K_m \) for Oxaloacetate** —Another interesting feature of the kinetic parameters of the 8S4D, S85I/R87Q/S88A, and G84D/S85I/R87Q/S88A mutated proteins is the correlation between the \( K_m \) values for the substrate (OAA) and for the cofactor. Whether the cofactor used is NADH or NADPH, the mutant with the lowest \( K_m \) for the cofactor also exhibits the lowest \( K_m \) for OAA. For example, compared with the wild type protein, the G84D mutated protein has a 10-fold lower \( K_m \) for NADH and also a 10-fold lower \( K_m \) for OAA. Similarly, the S85I/R87Q/S88A mutated protein has a 7-fold higher \( K_m \) for NADPH and a 6-fold higher \( K_m \) for OAA. This parallelism is even more striking in the G84D/S85I/R87Q/S88A mutated protein. A similar relationship has been reported for *T. flavus* MDH (20). This correlation suggests that the binding of the specific cofactor might facilitate substrate binding and, thus, that binding mechanism might be sequential, i.e. that the cofactor binds first, allowing subsequent OAA binding.

**Cofactor Specificity Is Correlated with the Specificity of the Inhibition of Activation** —In the absence of the cofactor in the activation medium, the classical slow activation kinetics of NADP-MDH are retained for the mutants having changed cofactor specificity (full activation within 10 min). When the oxidized cofactor is present in the activation medium, a parallel between the cofactor catalytic specificity and its ability to inhibit activation is observed. This effect is particularly striking with the G84D/S85I/R87Q/S88A mutant protein (Fig. 2), where cofactor specificity is totally reversed. This result confirms that in the oxidized enzyme, both oxidized and reduced forms of the cofactor bind to the same site. Previous observations showing that the inhibitory effect of NADP\(^+\) disappeared upon the elimination of the C-terminal disulfide (9) or the mutation of the penultimate C-terminal glutamate to a glutamine (5) indicated that the inhibitory effect was somehow associated with the C-terminal end of the enzyme and more specifically with the presence of the C-terminal disulfide. This inhibitory effect could not be accounted for by a direct effect of the oxidized cofactor on the redox potential of the C-terminal disulfide given that recent determinations of the redox potentials of the disulfides of NADP-MDH (15) showed that the oxidized cofactor did not have any significant effect on the redox potential required for the activation of the WT protein. Because it could be demonstrated that this potential reflects the redox potential of the most electronegative C-terminal disulfide, one could conclude that NADP\(^+\) binding does not affect the oxidation-reduction midpoint potential (\( E_{m'} \)) value of the C-terminal regulatory disulfide (15). Furthermore, as part of the current study, we have performed a determination of the redox potential of the
C-terminal disulfide of the SS51/R87Q/S88A mutant and did not find any difference between the redox potential of the WT enzyme and of the mutant within the ± 10 mV uncertainty of the measurement (~338 mV for the mutant, versus ~330 mV for the WT protein at pH 7; data not shown). In the light of the most recent structural data (6, 7), and of the aforementioned site-directed mutagenesis results, the effect of the oxidized cofactor can be better explained by its direct electrostatic interaction with the C-terminal extension and, more specifically, with its penultimate glutamate residue. Indeed, in the oxidized form of the enzyme, the C-terminal end is trapped inside the active site and thus located in close proximity of the positively charged nicotinamide ring of the cofactor (6, 7). Modeling the NAD\(^+\) position in our totally reversed cofactor specificity mutant (Fig. 3) suggests an identical situation in the mutant with the C termi

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