Communication

Direct NMR Observation of the Thioredoxin-mediated Reduction of the Chloroplast NADP-malate Dehydrogenase Provides a Structural Basis for the Relief of Autoinhibition*

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The chloroplastic NADP-dependent malate dehydrogenase (NADP-MDH) catalyzing the reduction of oxaloacetate into L-malate is regulated by light. Its activation results from the thioredoxin-mediated reduction of two disulfides, located, respectively, in N- and C-terminal sequence extensions typical of all NADP-dependent disulfides, located, respectively, in N and C-terminal results from the thioredoxin-mediated reduction of two disulfides, located, respectively, in N- and C-terminal extensions of typical all NADP-MDHs. Site-directed mutagenesis studies indicate this fact.

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Experimental Procedures

Materials—Restriction endonucleases, DNA modification enzymes, T4 DNA ligase, and T4 DNA polymerase were obtained from Appligene. DEAE-Sephadex and Matrix red A chromatographic supports were, respectively, from Amersham Pharmacia Biotech and Millipore. Chemicals (purchased from Sigma, Roche Molecular Biochemicals, or Pro- labo) were of analytical grade. Oligonucleotides were purchased from Eurogentec and Life Technologies, Inc. Radiolabels were from Amer- shack Pharamacia Biotech Escherichia coli strain XL1 blue (CLON- TECH) was used to produce high yields of plasmids and M13 single-stranded DNA. E. coli strain RZ1032 (Amersham Pharmacia Biotech) was used to produce dU-substituted M13 single-stranded DNA for site-directed mutagenesis. E. coli strain BL21 (DE3) (11) was used for the production of mutated NADP-MDHs encoded by recombinant plasmid vectors. M13 mp19 phage (Amersham Pharmacia Biotech) was used for site-directed mutagenesis and pET-8c (11) for the production of recombinant NADP-MDHs. Bacteria were grown at 37 °C on Luria broth medium; ampicillin at 50 μg/ml was added when the bacteria carried

Malate dehydrogenases (MDH) are homodimeric proteins of about 2 × 40 kDa that catalyze the interconversion of oxaloacetate to L-malate. The chloroplastic NADP-dependent enzyme (NADP-MDH (EC 1.1.1.82)) possesses the particularity to be regulated by light (1). The light activation is mediated by reduction of disulfide bridges through the ferredoxin-thiore- doxin system (2). Compared with the NAD-dependent MDHs, the amino acid sequences of the chloroplastic MDHs exhibit specific extensions at their N and C termini (3). In the S. Vulgaris enzyme activation by reduced thioredoxin is very fast compared with the wild-type enzyme. NMR measurements clearly show that a C-terminal inhibitory peptide would be released upon reduction, making the active site accessible (8). To better help understand the conformational rearrangements occurring during reduction, the structure of an active reduced enzyme would be most valuable. However, the need to use reduced thioredoxin to activate the enzyme makes crystallization of the active form difficult. As a first step toward a comprehensive structural representa-

the reduced forms of NADP-MDH were recorded. For this purpose, we have used an engineered MDH truncated in 33 residues at the N terminus. This truncated form is a monomer of 38 kDa (AN-MDH) still functional and redox-regulated, even though its activation by reduced thioredoxin is very fast compared with the wild-type enzyme. NMR measurements clearly show that a C-terminal peptide of 15 amino acids is released from the active site upon reduction and acquires an increased flexibility yielding locally narrow NMR line widths despite the relatively high molecular weight of the recombinant enzyme. Interactions be- tween the C-terminal extension residues and the core structure of the protein in the oxidized form as inferred from the crystal structure are discussed together with the NMR data obtained for the reduced form in order to explain the efficient reversible activation/inactivation redox control of the chloroplast MDH.
FIG. 1. Two-dimensional NMR spectra of the oxidized and reduced forms of the ΔN-MDH from *S. vulgar* (A). TOCSY spectrum of the oxidized ΔN-MDH. A', NOESY spectrum of the oxidized ΔN-MDH. B, TOCSY spectrum of the reduced ΔN-MDH. Boxes indicate cross-peaks found in the DQF-COSY spectrum. Sequential d_{ij} NOEs correlations are indicated with arrows. The sequence-specific assignment in the reduced enzyme is indicated. The amino acid numbering refers to the wild-type MDH from sorghum (8).

activation and enzyme activity assay—The activation kinetics of the MDH mutants were measured by preincubating the enzymes with 10 mM (±)-dithiothreitol and 20 μM recombinant thioredoxin (either from *E. coli* or from *Chlamydomonas reinhardtii*) in 100 mM Tris-HCl buffer, pH 7.9. Samples were taken at regular time intervals and injected into a spectrophotometer cuvette containing the reaction medium composed of 140 μM NADPH, 780 μM oxaloacetate in 100 mM Tris-HCl buffer, pH 7.9. The activity was recorded as a decrease in absorbance at 340 nm due to the oxidation of NADPH.

NMR samples—Oxidized ΔN-MDH NMR samples (550 μl) were prepared at 0.25–0.75 mM concentrations (10–30 mg/ml) in 20 mM phosphate buffer, 90% H_{2}O, 10% D_{2}O, pH 6.2. The proteins were reduced in the NMR cell under argon by addition of 10 μl of a 1 mM solution of thioredoxin h from *C. reinhardtii* (2.5–5 mol %, catalytic amount) (13) and 3 μl of a molar solution of (±)-dithiothreitol (Aldrich, 7–20 mol eq).

NMR experiments—All the NMR spectra were recorded using a Bruker Avance DRX 500 (1H = 500 MHz) spectrometer using a 5-mm sample tube, equipped with a supplementary self-shielded z gradient coil. Data were processed using the Bruker XWINNMR or GIFAV V.4.22 (14) software. All homonuclear two-dimensional experiments were recorded with 512 τ_{x} × 1024 (τ_{y}) complex data points (32 scans per τ_{y} increments). The data were apodized with shifted sine bell and gaussian window functions in both F_{1} and F_{2} dimensions after zero-filling in the τ_{y} dimension. The same window functions were applied for both redox states of the proteins. DQF-COSY (15), TOCSY (Hartmann-Hann spectroscopy) (16, 17), and NOESY (18, 19) experiments were recorded at 303 K. 1H chemical shifts were quoted relative to the solvent (H_{2}O) chemical shift at the temperature of the study (4.725 ppm at 303 K). The solvent signal was suppressed with the WATERGATE sequence using a 3-9-19 pulse sequence with 2 gradients (20, 21). For DQF-COSY an additional very low power presaturation of the water resonance during the relaxation delay to minimize the radiation damping effect. The quadrature detection in the τ_{y} dimension was achieved using the States-TPPI method (22). The spectral width of all experiments was 10 ppm (5000 Hz) with a carrier frequency on-resonance with the water resonance. For TOCSY experiments, a MLEV pulse sequence was used for the isotropic mixing, arranged according to the clean-TOCSY method (23) to minimize the signals arising from the rotating frame Overhauser effect. In the NOESY experiment, a water flip back pulse was used and gradients were added during τ_{y} to minimize the radiation damping effect (24). The total mixing times were 40 ms for TOCSY spectra and 75 and 150 ms for NOESY spectra.

RESULTS AND DISCUSSION

Biochemical activity of the ΔN-MDH—The chloroplastic NADP-dependent malate dehydrogenase is a homodimeric enzyme activated by the reduction of two disulfide bridges located at the N and C termini of each monomer. Its activation rate is slow, with a distinct lag phase. The truncated ΔN-MDH is a monomer devoid of the N-terminal disulfide but still totally inactive in the oxidized form. After activation, its catalytic properties are identical to those of the WT enzyme (5). It constitutes a simplified system where the effect of the C-terminal extension can be studied independently from the interaction with the N-terminal extension. In addition, the 38kDa molecular mass of the monomeric ΔN-MDH makes it potentially accessible to high resolution NMR in solution (25), allowing more detailed future structural studies.

It has been shown previously that this mutant exhibits fast activation kinetics, suggesting that the reduction of the N-terminal disulfide was the rate-limiting step of the activation. This N-terminal extension is also necessary for the dimerization (6). For the WT dimeric enzyme, site-directed mutagenesis
and crystallographic studies clearly demonstrated that the C-terminal extension was bound inside the active site, acting as an internal inhibitor in the oxidized enzyme (Fig. 2) (8, 10). It could be expected that upon reduction by thioredoxin, the C-terminal inhibitory extension should be released from the active site. The fast activation rate of the mutants missing the N-terminal disulfide bridge suggested that a local shift of the active site. The fast activation rate of the mutants missing the N-terminal disulfide bridge should be expected that upon reduction by thioredoxin, the C-terminal extension was bound inside the active site, acting as an internal inhibitor in the oxidized enzyme (Fig. 2) (8, 10). It could be expected that upon reduction by thioredoxin, the C-terminal inhibitory extension should be released from the active site. The fast activation rate of the mutants missing the N-terminal disulfide bridge suggested that a local shift of the active site.

**NMR Spectra of Reduced and Oxidized Forms of \( \Delta N \)-MDH and the C207A \( \Delta N \)-MDH Mutant—**From the crystallographic structure of the oxidized MDH (Fig. 2), it was proposed that the reduction of the C-terminal Cys365–Cys377 disulfide bridge would release the last two C-terminal residues Glu388 and Val389 from the active site, making it accessible to the substrate. This unblocking of the MDH active site could be the consequence of an increased mobility of the C-terminal extension upon reduction of the Cys365–Cys377 disulfide bridge by thioredoxin. Such a mobility difference could be characterized by the comparison of both redox states of the chloroplast MDH by solution NMR which is sensitive to molecular motions.

The NMR spectra of the oxidized form of the \( \Delta N \)-MDH and the \( \Delta N \)-MDH C207A mutant indicate no COSY cross-peaks and the C207A mutant is a good alternative to the use of the oxidized MDH (Ref. 8; 7MDH entry of the Protein Data Bank) from *S. vulgare*, showing the C-terminal disulfide, the residues of the active site (Asn173, Asp201, Arg204, and His229, and the C-terminal inhibitory peptide. B, amino acid sequence of the C-terminal extension of the MDH from *S. vulgare* from the first cysteine of the C-terminal disulfide. The peptide observed in the NMR spectra of the reduced form of the enzyme is shown in gray. The amino acid numbering refers to the wild-type MDH from sorghum (8).

Table I

| Peptide sequences identified from the two-dimensional spectra of the reduced \( \Delta N \)-MDH | Possibilities in the protein (residues 1–364) | Possibilities in the C-terminal (residues 365–389) |
|-----------------|-----------------|-----------------|
| A Y(AMX)(AMX)   | No AX           | AXCD            |
| G(E/M/Q)/V      | GEV             | GEV             |
| T(E/M/Q)/L      | /               | TML             |

Table II

| 1H chemical shifts (ppm) of the C-terminal peptide observed in the reduced \( \Delta N \)-MDH at 303 K |
|-----------------------------------------------|
| Residue | NH | aH | pH | Others |
|---------|----|----|----|--------|
| Ala375  | 8.18 | 4.22 | 1.24 | γCH2 7.10; δCH2 6.80 |
| Tyr376  | 8.08 | 4.52 | 2.95 | 2.93 |
| Cys377  | 8.00 | 4.43 | 2.82 | 2.82 |
| Asp378  | 8.36 | 4.60 | 2.67 | 2.67 |
| Val379  | 8.01 | 4.39 | 2.05 | 2.05 |
| Pro380  | 8.04 | 4.38 | 2.38 | 2.38 |
| Gly381  | 8.18 | 4.22 | 1.24 | 1.24 |
| Cys382  | 8.00 | 4.43 | 2.82 | 2.82 |
| Glu383  | 8.15 | 4.32 | 1.92 | 1.92 |
| Val384  | 7.68 | 4.03 | 2.05 | 2.05 |
| Thr385  | 7.68 | 4.03 | 2.05 | 2.05 |
| Met386  | 7.68 | 4.03 | 2.05 | 2.05 |
| Leu387  | 7.68 | 4.03 | 2.05 | 2.05 |
| Asp388  | 8.30 | 4.55 | 2.62 | 2.62 |
| Tyr389  | 8.08 | 4.52 | 1.92 | 1.92 |
| Glu390  | 8.15 | 4.32 | 1.92 | 1.92 |

a Not assigned.

Recent experiments have shown that in the presence of thiol oxidants the \( \Delta N \)-MDH could form dimers through an intersubunit disulfide bridge linking the Cys207 of both subunits (12). Therefore, a C207A mutant of \( \Delta N \)-MDH, unable to form covalent dimers, was also studied, in order to avoid potential dimerization problems. This mutant is a good alternative to the use of the \( \Delta N \)-MDH, having similar activation properties and catalytic parameters (data not shown).

FIG. 2. C-terminal inhibitory peptide of the oxidized MDH from *S. vulgare*. A, representation of a monomer of the inactive-oxidized MDH (Ref. 8; 7MDH entry of the Protein Data Bank) from *S. vulgare*, showing the C-terminal disulfide, the residues of the active site (Asn173, Asp201, Arg204, and His229, and the C-terminal inhibitory peptide. B, amino acid sequence of the C-terminal extension of the MDH from *S. vulgare* from the first cysteine of the C-terminal disulfide. The peptide observed in the NMR spectra of the reduced form of the enzyme is shown in gray. The amino acid numbering refers to the wild-type MDH from sorghum (8).
C-terminal peptide Ala^{375}-Val^{389}, the last 15 residues of the protein (Tables I and II and Fig. 2). Therefore, it is clear that the C-terminal peptide of the enzyme is no longer bound to the core structure of the protein when the C-terminal Cys^{365}-Cys^{377} disulfide is reduced. The relative intensities of the sequential $d_{35}^{20}$ and $d_{35}^{20}$NOEs and the absence of any $(i, i + 2)$ or longer distance NOEs, correlate with a random-coil conformation for the C-terminal peptide of the enzyme. The transverse relaxation times $T_2$ of the protons located in the C-terminal peptide were gained significantly by the actual additional motion, leading to observable magnetization transfers in the different two-dimensional NMR experiments.

**Structural Rearrangements of the C-terminal Part during the Activation Process**—The assignment of the mobile peptide provides information about the structural rearrangements occurring upon reduction. The first part of the C-terminal peptide from Cys^{365} to Asn^{374} remains bound to the core structure as the corresponding residues keep a correlation time similar to that of the rest of the protein. The peptide is mobile from Ala^{375}, i.e. only two residues before the second cysteine of the regulatory C-terminal disulfide Cys^{365}-Cys^{377}. This is probably a favorable feature to maintain the Cys^{377} not too far away from Cys^{365}, and the rest of the inhibitory C-terminal peptide not too far away from the whole protein. By this means, the inhibitory C-terminal peptide will easily find its way back to the active site when the photosynthetic electron transfer is stopped in the dark. This will allow MDH to readily reoxidize and consequently self-inactivate.

The conformational analysis of the C-terminal extension in the inactive, oxidized crystalline form of the enzyme (8) supports the assumption that the structure of the Cys^{365}-Asn^{374} part is not strongly modified in the reduced active form in solution. Residues 365 and 366 are part of the last helix of the protein. The other amino acids from positions 367 to 374 do not form a regular secondary structure but are stabilized by a number of hydrogen bonds (O-366 with N-369 and N-370, O-367 with N-371 and N-372, O-364 with N-376, side chain of Thr^{370} and N-372, O-369 with side chain of Asn^{364} and side chain of His^{365} with side chain of Asp^{145}). This indicates that residues 371-374 are more tightly bound to the protein than the rest of the C-terminal peptide. A number of interactions constrain the rest of the C-terminal extension to fold near the core structure of the protein in addition to the covalent interaction due to the disulfide bridge. In particular, Val^{375}, Met^{384}, and Leu^{385} make hydrophobic interactions with residues of the rest of the protein and a number of hydrogen bonds are found between the peptide and the protein. We should assume that these interactions are not sufficient to hold the C terminus in the active site in the reduced form. However, these interactions undoubtedly stabilize the binding to the core structure when the C-terminal disulfide is oxidized.

Our work provides the first direct experimental observation of the in vitro thioredoxin-mediated activation of a thiol-regulated plant MDH. It shows that the activation is the result of the unobstruction of the active site by the acquisition of an additional mobility of the most C-terminal 15-residue stretch.

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