



RESEARCH PAPER

Self-protection of cytosolic malate dehydrogenase against oxidative stress in Arabidopsis

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Abstract

Plant malate dehydrogenase (MDH) isoforms are found in different cell compartments and function in key metabolic pathways. It is well known that the chloroplastic NADP-dependent MDH activities are strictly redox regulated and controlled by light. However, redox dependence of other NAD-dependent MDH isoforms have been less studied. Here, we show by *in vitro* biochemical characterization that the major cytosolic MDH isoform (cytMDH1) is sensitive to H₂O₂ through sulfur oxidation of cysteines and methionines. CytMDH1 oxidation affects the kinetics, secondary structure, and thermodynamic stability of cytMDH1. Moreover, MS analyses and comparison of crystal structures between the reduced and H₂O₂-treated cytMDH1 further show that thioredoxin-reversible homodimerization of cytMDH1 through Cys330 disulfide formation protects the protein from overoxidation. Consistently, we found that cytosolic thioredoxins interact specifically with cytMDH in a yeast two-hybrid system. Importantly, we also show that cytosolic and chloroplastic, but not mitochondrial NAD-MDH activities are sensitive to H₂O₂ stress in Arabidopsis. NAD-MDH activities decreased both in a catalase2 mutant and in an NADP-thioredoxin reductase mutant, emphasizing the importance of

Abbreviations: CD, circular dichroism; chlMDH, chloroplastic NAD-dependent MDH; cytMDH, cytosolic NAD-dependent MDH; IAM, iodoacetamide; MDH, malate dehydrogenase; mitMDH, mitochondrial NAD-dependent MDH; NADP-MDH, NADP-dependent MDH; NTR A/B, NADPH-dependent thioredoxin reductase A/B; OAA, oxaloacetate; perMDH, peroxisomal NAD-dependent MDH; SEC, size exclusion chromatography; -SOH, sulfenic acid; TCA, tricarboxylic acid; TRX, thioredoxin.

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the thioredoxin-reducing system to protect MDH from oxidation *in vivo*. We propose that the redox switch of the MDH activity contributes to adapt the cell metabolism to environmental constraints.

Keywords: CytMDH1, dimerization, H₂O₂-triggered oxidation, overoxidation, sulfenic acid, thioredoxin.

Introduction

NAD(P)-dependent malate dehydrogenases (MDHs, L-malate:NAD oxidoreductases; EC 1.1.1.37) reversibly catalyze the oxidation (dehydrogenation) of L-malate to oxaloacetate (OAA) while reducing NAD(P)⁺ to NAD(P)H. In plants, MDH isoforms play a role in central metabolism and redox homeostasis between organellar compartments (Faske *et al.*, 1995; Scheibe, 2004; Scheibe and Dietz, 2012; Selinski *et al.*, 2014). As in other plants, Arabidopsis MDH isoforms can be classified into different groups based on their localizations: three cytosolic NAD-dependent MDHs, cytMDH1, cytMDH2, and cytMDH3; two mitochondrial NAD-dependent MDHs, mitMDH1 and mitMDH2; one chloroplastic NAD-dependent MDH, chlMDH; two peroxisomal NAD-dependent MDHs, perMDH1 and perMDH2; and one NADP-dependent MDH (NADP-MDH) (see Supplementary Fig. S1 at JXB online). NADP-MDH, localized in the chloroplast, is redox regulated through two sets of redox-sensitive cysteine pairs, located at the N- and C-terminus, which form two intramolecular disulfides (Cys77–Cys82 and Cys418–Cys430) that inhibit the enzymatic activity (Issakidis *et al.*, 1992, 1994; Faske *et al.*, 1995; Ruelland *et al.*, 1998; Johansson *et al.*, 1999; Carr *et al.*, 1999). The inactive disulfide-containing state of NADP-MDH can be reduced to its active form through thiol/disulfide exchange with specific classes of disulfide reductases, namely thioredoxins (TRXm and TRXf), which themselves are reduced by a light-dependent ferredoxin-TRX reductase (reviewed by Jacquot *et al.*, 1997; Lemaire *et al.*, 2007; Meyer *et al.*, 2009). Active NADP-MDH oxidizes NADPH to convert OAA into malate. Malate is then transported to the cytosol, where the cytMDH converts it back to OAA, while reducing NAD⁺ to NADH in the process. This so-called ‘malate valve’ is the first step in the redistribution of chloroplastic reducing equivalents to other parts of the plant cell (Scheibe, 2004; Hossain and Dietz, 2016). Two decades ago, chlMDH was reported as a non-redox-regulated protein (Berkemeyer *et al.*, 1998), and recently mitMDH was shown to be redox independent (Yoshida and Hisabori, 2016). As the redox-sensitive cysteine pairs of the NADP-MDH are absent in cytMDH isoforms, it had not been considered as a redox-sensitive enzyme. However, cytMDH was captured on a TRX affinity chromatography column (Yamazaki *et al.*, 2004), and found to be S-nitrosothiolated upon gaseous nitric oxide treatment in Arabidopsis (Lindermayr *et al.*, 2005). More recently, cytMDH was detected in a sulfenylated state (–SOH) in *Medicago truncatula* (Oger *et al.*, 2012), and in Arabidopsis cell suspensions (Waszczak *et al.*, 2014; Akter *et al.*, 2015). In an *in vitro* study, CuCl₂ treatment was shown to inactivate cytMDH and a disulfide-linked homodimer was

formed. Moreover, this disulfide bond was efficiently reduced by a cytosolic thioredoxin h1 (TRXh1) (Hara *et al.*, 2006).

In Arabidopsis, cytosolic TRXs are encoded by multigenic families for which TRXh members are the major isoforms (reviewed by Meyer *et al.*, 2009). Most of these members are reduced by NADPH-dependent TRX reductases (NTRA and NTRB) that are dually located in the cytosol and mitochondria (Laloi *et al.*, 2001; Reichheld *et al.*, 2005). Some discrete TRXh members are alternatively reduced by the glutathione reductase (GR)/glutaredoxin (GRX) pathway, another major cytosolic thiol reduction pathway dependent on NADPH (Gelhaye *et al.*, 2003).

Here, we re-examined the redox dependence of cytMDH by combining *in vitro* biochemical, structural, and biophysical approaches. We reveal that oxidation triggers overoxidation of several cysteine residues through sulfenylation. Moreover, we show that under oxidative stress, cytMDH switches from a non-covalent dimer to a covalent disulfide-linked dimer, probably affecting the structure, the kinetics, and the stability of the enzyme. We indicate that the dimerization via Cys330–Cys330 disulfide acts as a redox switch, protecting cytMDH1 from overoxidation, and can be reduced by TRXs. Importantly, we show that such regulation also occurs *in planta* and might be conserved in other cytMDH isoforms. Therefore, based on both *in vitro* and *in planta* data, we propose a mechanism of how cytosolic NAD-MDH is redox regulated.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana plants were grown in soil in a controlled growth chamber (180 µE m^{−2} s^{−1}, 16 h day/8 h night, 22 °C 55% relative humidity day, 20 °C 60% relative humidity night) for up to 3 weeks. Plant mutant lines *ntra ntrb*, *cat2*, *gr1*, and *gr1 cat2* were previously described (Queval *et al.*, 2007; Reichheld *et al.*, 2007; Mhamdi *et al.*, 2010a).

In vitro protein-based complementation and TRX activity assays

For *in vitro* protein-based complementation assays, 5 µg of protein extracts (200 ng µl^{−1}) were incubated with 1 mM NADPH, 4.59 µM TRXh3, and 3.12 µM NTRA in 25 µl 50 mM Tris–HCl (pH 7.5) for 2 h on ice. This reaction mixture was diluted 40 times in the same buffer, and the NAD-MDH activity assay was performed as described above.

Yeast two-hybrid assay

For yeast expression, full-length TRXh and MDH cDNA were respectively cloned in the pDEST32 and pDEST22 plasmids using the Gateway cloning system. Yeast two-hybrid assays were performed

according to the method described previously (Vignols *et al.*, 2005). Briefly, the yeast strain CY306 (a *Δtrx1Δtrx2* mutant) was co-transformed with both pDEST22 and pDEST32 constructs and plated on YNB medium with the required amino acids and bases (His, Ura, Lys, Ade, and Met). Transformed colonies were subcultured and diluted using dilution series (diluted at 5×10^{-2} , 5×10^{-3} , and 5×10^{-4} at an optical density of 600 nm) prior to dotting on YNB –His –Trp –Leu in Petri dishes containing 20 mM 3-amino-1,2,4-triazole (3AT). Three independent transformations per binary assay were performed, of which 2–4 colonies were assayed. Images were taken 4 d post-dotting.

Cloning, expression, and purification of recombinant cytMDH1

The cytMDH1-coding sequence (Atlg04410), with attL and PreScission (EVLFG/GP) sites at the N-terminal end, was synthesized by the Gen9 company. The gene fragment was inserted into the pDEST17 vector (Life Technologies). The constructs were transferred into *Escherichia coli* C41(DE3) strain.

The expression and purification of recombinant His-tagged cytMDH1 was done as previously described (Hara *et al.*, 2006). The purified His-tagged cytMDH1 was incubated with PreScission HRV3C enzyme (20:1, w/w) at 4 °C overnight to remove the His tag. The cleaved protein sample was loaded onto an Ni²⁺-Sepharose column equilibrated with 50 mM Tris–HCl, pH 7.5, 200 mM NaCl. The flow-through was collected and evaluated on an SDS–polyacrylamide gel as pure cleaved recombinant protein. Purified protein samples were pooled and flash-frozen in liquid nitrogen and then stored at –80 °C.

Protein extracts from Arabidopsis plants and cytMDH1 enzymatic assay

Purified cytosolic, mitochondrial, and chloroplastic protein extracts were prepared as described before (Daloso *et al.*, 2015). NAD-MDH activity was measured from 3–5 μg of protein extracts at 25 °C in a reaction medium containing 50 mM Tris–HCl (pH 7.5), 250 μM NADH, and 2.5 mM OAA. Catalase activity was measured for 20 μg of protein extracts at 25 °C in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.0) and 10 mM H₂O₂. The activity was monitored as a change in absorbance at 240 nm due to breakdown of H₂O₂.

Purified recombinant cytMDH1 protein was incubated with 20 mM DTT at room temperature for 1 h to obtain reduced cytMDH1 (cytMDH1_{Red}). cytMDH1_{Red} (8 μM) was incubated with 10 mM H₂O₂ at 30 °C for 45 min to obtain oxidized cytMDH1 (cytMDH1_{Ox}).

For oxidation by diamide, 8 μM cytMDH1_{Red} was incubated with 40 μM or 1 mM diamide at 30 °C for 45 min. For oxidation of the iodoacetamide (IAM)-alkylated cytMDH1, 20 μM cytMDH1_{Red} was incubated with 10 mM IAM at room temperature for 20 min. Then 8 μM cytMDH1_{IAM} was incubated with 10 mM H₂O₂ at 30 °C for 45 min.

After each treatment, the protein samples were passed through a micro bio-spin column (Bio-Rad) to remove chemicals. The concentration of modified cytMDH1 was measured by absorbance at 280 nm. For cytMDH1 enzymatic assay, the reaction was performed in a 96-well plate at 30 °C with a final volume of 200 μL (ThermoFisher Scientific), containing 50 mM Tris–HCl pH 7.5, 2.5 mM OAA, 0.25 mM NADH, and 2.5 nM cytMDH1. The decrease in NADH absorbance at 340 nm was monitored using a SpectraMax 340PC spectrophotometer (Molecular Devices). The molar extinction coefficient for NADH of 6220 M^{–1} cm^{–1} was used for the calculation. To obtain the K_M for OAA or NADH, progress curves were recorded using varying concentrations of OAA (0–2.5 mM) or NADH (0.0125–0.25 mM). The initial velocity (v_i) for each substrate concentration was measured, and the v_i/E_0 values were plotted and fitted with the Michaelis–Menten equation to obtain the kinetic parameters. Three independent replicates of v_i were measured for each substrate concentration. For the time course experiment with H₂O₂ incubation, 4.5 μM cytMDH1_{Red} was incubated with

50 mM Tris–HCl (pH 7.5)/10 mM DTT/10 mM H₂O₂ at 30 °C for 2 h. CytMDH1 was diluted to 2.5 nM to measure its activity. For MDH reactivation by TRXh, cytMDH1_{Ox} was incubated with the indicated concentrations of recombinant TRXh in the presence of NADPH (0.125 mM) and NTRA (0.5 μM). After 30 min incubation, the mix was diluted 40-fold and used for the NAD-MDH activity assay, as described above.

In vitro sulfenylation

Dimedone (500 mM; Sigma-Aldrich) was prepared freshly in DMSO. Then 8 μM cytMDH1_{Red} was incubated with 2 mM dimedone in the presence of 10 mM DTT or 10 mM H₂O₂ at 30 °C for 45 min. The excess was removed on an equilibrated Micro Bio-Spin® Column Bio-Gel® P-6. The eluted samples were treated with 2 mM IAM at room temperature for 20 min. The samples were run on an SDS–polyacrylamide gel and the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane. The western blot was done with 1:10 000 dilution rabbit anti-Cys-SOH antibody (Millipore); the result was visualized using Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate (PerkinElmer).

LC–MS/MS

The dimedone-treated cytMDH1_{Red} and cytMDH1_{Ox} samples were examined by SDS–PAGE and the bands were excised after Coomassie Brilliant Blue (CBB) staining. After destaining, the protein samples were digested by trypsin and subjected to LC–MS/MS analysis.

The peptides were analyzed by LC–MS/MS as described (Pyr Ditt Ruys *et al.*, 2012). The mass spectrometer was operated in the data-dependent mode and switched automatically between MS, Zoom Scan for charge state determination and MS/MS for the most abundant ion. Each MS scan was followed by a maximum of five MS/MS scans using a collision energy of 30%. Dynamic exclusion was enabled to allow analysis of co-eluting peptides. The following parameters were used: trypsin was selected with cleavage only after lysine and arginine; the number of internal cleavage sites was set to 1; the mass tolerance for precursor and fragment ions was 1.1 Da and 1.0 Da, respectively; and the considered dynamic modifications on cysteine residues were +138.0 Da for sulfenic-dimedone, +32.0 Da for sulfinic, +48.0 Da for sulfonic, and +57.0 Da for carbamidomethyl modifications. For peptide identification, peak lists were generated using the application spectrum selector in the Proteome Discoverer 1.4 package. The resulting peak lists were searched using Sequest against an *Arabidopsis* protein database. Peptide matches were filtered using Percolator within Proteome Discoverer and manually validated. The mixed disulfide peptide between C330 and C330 of MDH was identified by the use of DBond software (Choi *et al.*, 2010). All the annotated MS/MS spectra of modified peptides were manually evaluated.

Melting and overall secondary structure assessed by circular dichroism (CD)

The far CD spectra for cytMDH1_{Red} and cytMDH1_{Ox} were recorded in a JASCO J-715 spectropolarimeter equipped with a Peltier temperature control unit. The measurements were carried out in a 0.1 cm path length cuvette with an average of three scans for each spectrum, a 100 nm min^{–1} scan speed, band width of 2 nm, data pitch of 4 nm s^{–1}, and the recorded range was from 190 nm to 260 nm. The buffer used was 50 mM ammonium bicarbonate, pH 7.4, and the protein concentration was in the range of 3–4.5 μM. Thermal unfolding was carried out at 4.5 μM protein concentration by increasing the temperature of the cuvette from 15 °C to 75 °C, and monitoring the intensity peak shift at 222 nm. Thermodynamic parameters and melting temperature of cytMDH1_{Red} and cytMDH1_{Ox} unfolding were calculated as described previously (Suh and Savitsky, 2011). The melting temperature was calculated assuming that the unfolding process occurred following a simple two-state model. The peak

shift at 222 nm over the specified temperature range was fitted into the following dose–response equation:

$$y = A + \frac{Z - A}{1 + 10^{(\log X_0 - X) \cdot h}} \quad (1)$$

The terms of Equation 1 are as follows: A is the folded state, Z is the unfolded state, $\log X_0$ is the melting temperature, and h is the Hill coefficient. The thermodynamic parameters were estimated based on the following equations:

$$\Delta G = 2.303 \times R \times h \times (T \times T_m - T^2) \quad (2)$$

$$\Delta H = 2.303 \times R \times T^2 \times h \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

where ΔG is the free energy Gibbs variation, ΔH is the enthalpy variation, ΔS is the entropy variation and T is the temperature (30 °C). Protein reduction and oxidation were performed as described before.

Crystallization, X-ray data collection, and structure solution

Crystals of native cytMDH1 were grown by hanging-drop vapor diffusion at 20 °C in droplets with 1:1 addition of 18 mg ml⁻¹ cytMDH1 to a precipitant of 0.18 M ammonium sulfate, 0.09 M sodium acetate trihydrate, pH 4.6, 27% polyethylene glycol monomethyl ether 2000, and 10% glycerol. Crystals intended for oxidation were also obtained by hanging-drop vapor diffusion, with a crystallization solution of 1.5 M ammonium sulfate, 0.1 M Tris–HCl, pH 8.0. Crystallization droplets were composed of 1 µl of cytMDH1 and 1 µl of precipitant solution, with NAD⁺ present in 10× molar excess.

For X-ray data collection, native cytMDH1 crystals were cryoprotected by supplementation of 80% mother liquor with 20% ethylene glycol. Diffraction data were collected using an in-house MicroMax-007HF X-ray generator with a Saturn 944+ detector. Crystals of cytMDH1 were oxidized through inclusion of 30 mM H₂O₂ in the cryoprotectant of 0.75 M ammonium sulfate, 50 mM Tris–HCl, pH 8.0, 16% ethylene glycol, 10% glycerol, 10% 1,2-propanediol, with a soak duration of 5 min. Diffraction data from these H₂O₂-treated crystals were collected at the Proxima 2a beamline at SOLEIL synchrotron (<https://www.synchrotron-soleil.fr/en>).

Indexing and integration of reflections was performed in XDS (Kabsch, 2010a, b), and scaling and merging using AIMLESS (Evans and Murshudov, 2013). Initial phases were determined in Phaser (McCoy *et al.*, 2007) of the PHENIX software suite (Adams *et al.*, 2002, 2010) with *Sus scrofa* MDH as search model (PDB ID: 4MDH, 62% sequence identity). Manual model building was performed in COOT (Emsley and Cowtan, 2004), with maximum-likelihood refinement in Phenix.refine (Afonine *et al.*, 2012), and REFMAC5 (Murshudov *et al.*, 2011) of the CCP4 suite (Collaborative Computational Project, 1994). Structural stereochemistry was checked in MolProbity (Chen *et al.*, 2010). The native and peroxide-treated crystal structures of cytMDH1 have been submitted to the PDB under the accession codes 5NUF and 5NUE, respectively. Statistics of data reduction and refinement are summarized in Supplementary Table S3. Biomolecular interfaces were analyzed, and biological assemblies assigned using the PISA web service (Krissinel and Henrick, 2007). All structural figures were prepared in PyMOL.

Accession numbers

Assigned accession numbers for the genes used in this work are as follows: At1g04410 (cytMDH1), At5g43330 (cytMDH2), At5g56720 (cytMDH3), At1g53240 (mtMDH1), At3g15020 (mtMDH2), At2g22780 (perMDH1), At5g09660 (perMDH2), At3g47520

(chlMDH), At5g58330 (NADP-MDH). Reduced and oxidized cytMDH1 structures were deposited at the PDB database (www.PDB.org) with the respective IDs: 5NUF and 5NUE.

The RNA sequencing (RNA-Seq) (Waszczak *et al.*, 2016) and microarray data (Willems *et al.*, 2016) discussed in this article have been deposited in the GEO repository (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through the GEO accession nos GSE77017 and GSE80200, respectively.

Results

H₂O₂-treated cytMDH1 forms a disulfide-linked homodimer

In order to characterize redox modifications of cytMDH1, we produced cytMDH1 in *E. coli*, and purified the protein to homogeneity. To validate cysteine sulfonylation *in vitro*, we applied dimedone, a chemical compound that forms a thioether bond with the electrophilic sulfur of sulfonylated proteins. CytMDH1_{Red} migrated as an ~37 kDa band, which is very close to the expected size of 35.7 kDa based on the amino acid sequence. However, we detected an additional ~70 kDa band of cytMDH1_{Ox} (10 mM H₂O₂) on the non-reducing CBB-stained SDS–PAGE gel (Fig. 1A), suggesting that cytMDH1 forms a covalently linked dimer upon oxidation. On immunoblots with anti-dimedone antibodies (Fig. 1A), we only observed sulfonylation of the ~37 kDa band of cytMDH1_{Ox}. Remarkably, the dimer was not sulfonylated, suggesting that the covalent dimerization protected cytMDH1_{Ox} from cysteine sulfonylation. We further studied whether the covalent dimer and the sulfonylated form were reduced by DTT and/or the NADPH-dependent TRX system (NTS). While the covalent homodimer was readily reduced either by DTT or NTS, consistent with previous data of Hara *et al.* (2006), the sulfonylated MDH monomer was only could be reduced by DTT (Fig. 1B).

To confirm the molecular size of the reduced and oxidized cytMDH1, we evaluated their elution position on a size exclusion chromatography (SEC) column (Supplementary Fig. S2). Both cytMDH1_{Red} and cytMDH1_{Ox} eluted as a single peak of ~70 kDa, indicating that both cytMDH1_{Red} and cytMDH1_{Ox} are in a dimeric form in solution. We further examined cytMDH1 dimerization by migrating cytMDH1_{Red} and cytMDH1_{Ox} on native gels (Supplementary Fig. S3). Both cytMDH1_{Red} and cytMDH1_{Ox} migrated at an apparent molecular mass of ~100 kDa in either non-reducing or reducing conditions. The observed molecular mass is slightly higher than that expected for a dimer (~72 kDa), which could be due to the charge of the protein. However, consistent with the SEC data, no monomeric forms were observed under reducing or oxidizing conditions, supporting the fact that both dimeric forms co-exist (covalent and non-covalent) depending on the redox environment of the protein. cytMDH1_{Red} dimer is probably organized by non-covalent interactions, while cytMDH1_{Ox} is likely to be a mixture of non-covalent and covalent dimers (Fig. 1; Supplementary Figs S2, S3).

We also detected several bands close to each other in cytMDH1_{Ox} on the native gel (Supplementary Fig. S3), probably

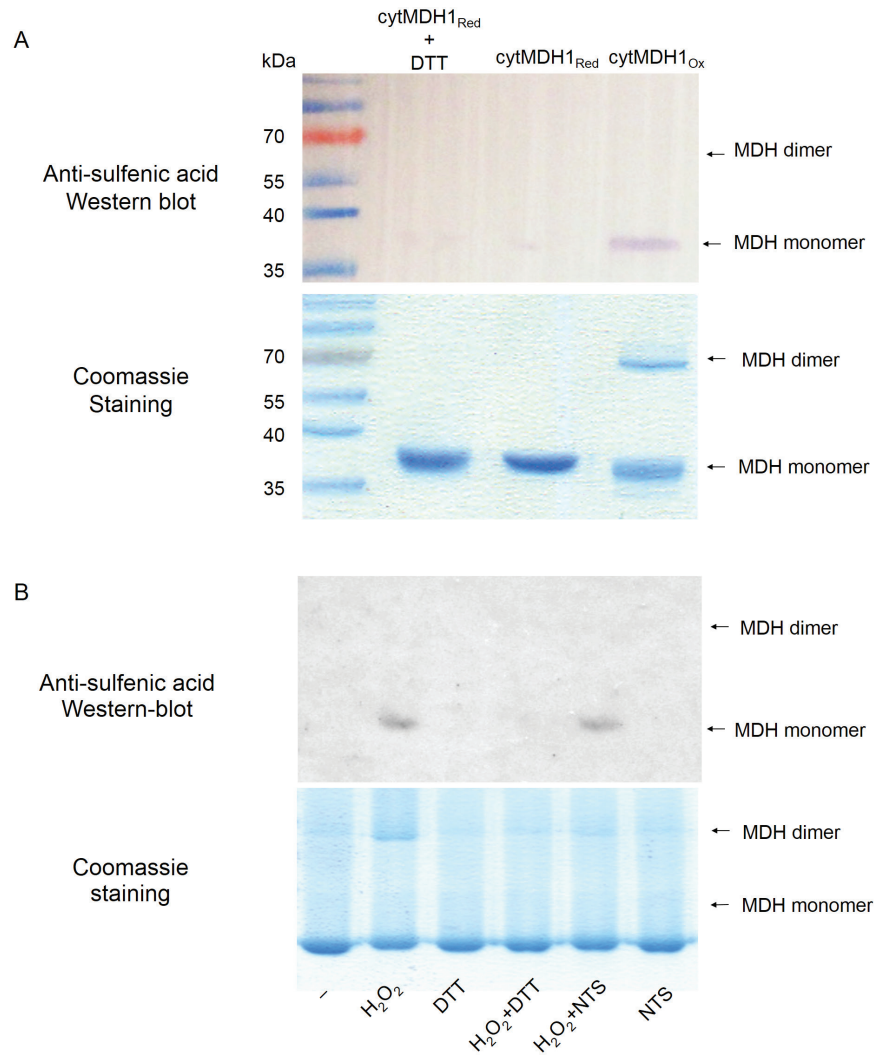


Fig. 1. cytMDH1_{Ox} is partially sulfenylated *in vitro* and dimerizes via a TRX-reducible intermolecular disulfide bond. (A) cytMDH1_{Red} (8 μ M) was incubated with 10 mM DTT (cytMDH1_{Red}+DTT) or treated with 10 mM H₂O₂ (cytMDH1_{Ox}) in the presence of 2 mM dithionite. Samples were analyzed by SDS-PAGE followed by Coomassie blue staining. cytMDH1-SOH formation was analyzed on a western blot with an anti-cysteine sulfenic acid antibody. (B) cytMDH1_{Red} (8 μ M) was incubated with 10 mM DTT (cytMDH1_{Red}+DTT) or treated with 10 mM H₂O₂ (cytMDH1_{Ox}) in the presence or absence of the NTS system. Samples were analyzed by SDS-PAGE followed by Coomassie blue staining. cytMDH1-SOH formation was analyzed by immunoblot with an anti-cysteine sulfenic acid antibody.

due to oxidation of other cysteines, which gives extra charges to the protein. Notably, the upper band (in between 100 kDa and 130 kDa) almost completely disappeared under reducing conditions. Whether this form corresponds to the covalent dimer cannot be fully confirmed.

Collectively, our data suggest that cytMDH1 undergoes a partial conformational change from a non-covalent homodimer to a disulfide-linked homodimer under H₂O₂ stress.

Cysteine and methionine sulfurs are sensitive to oxidation

To confirm the sulfenylation of cytMDH1 *in vitro* and the disulfide bond formation of cytMDH1_{Ox}, we analyzed the purified recombinant protein treated with or without H₂O₂ in the presence of dithionite by MS. After treatment with 10 mM H₂O₂ and 2 mM dithionite, we blocked all free thiols by IAM, and the cytMDH_{Ox} sample was loaded on a non-reducing SDS-polyacrylamide gel. The 37 kDa and 70 kDa

bands (Fig. 1A) were in-gel trypsin digested prior to MS analysis. In the 37 kDa band, a dithionite adduct on the Cys330-containing peptide was found, resulting in a mass increase of 138 Da (Fig. 2A), showing that Cys330 was sulfenylated. Cys330 was also partially present as a free thiol (carbamidomethyl modification by IAM) (Supplementary Table S1) and overoxidized to a sulfonic acid (Fig. 2B). Furthermore, Cys79 was also sulfenylated and overoxidized to a sulfenic acid and sulfonic acid (Supplementary Table S1). All other cysteines were identified as free thiols (Supplementary Table S1). In the 70 kDa band, an intermolecular disulfide bond between Cys330 was found (Fig. 2C), as described previously (Hara *et al.*, 2006). Besides these cysteine modifications, we also observed that methionines were oxidized to a methionine sulfoxide in both 35 kDa (Met24/30/56/87/313) and 70 kDa (Met24/30/41/56/87/270/313) bands (Supplementary Table S1). Among these oxidized methionines, Met313 had been identified previously as a methionine sulfoxide in the

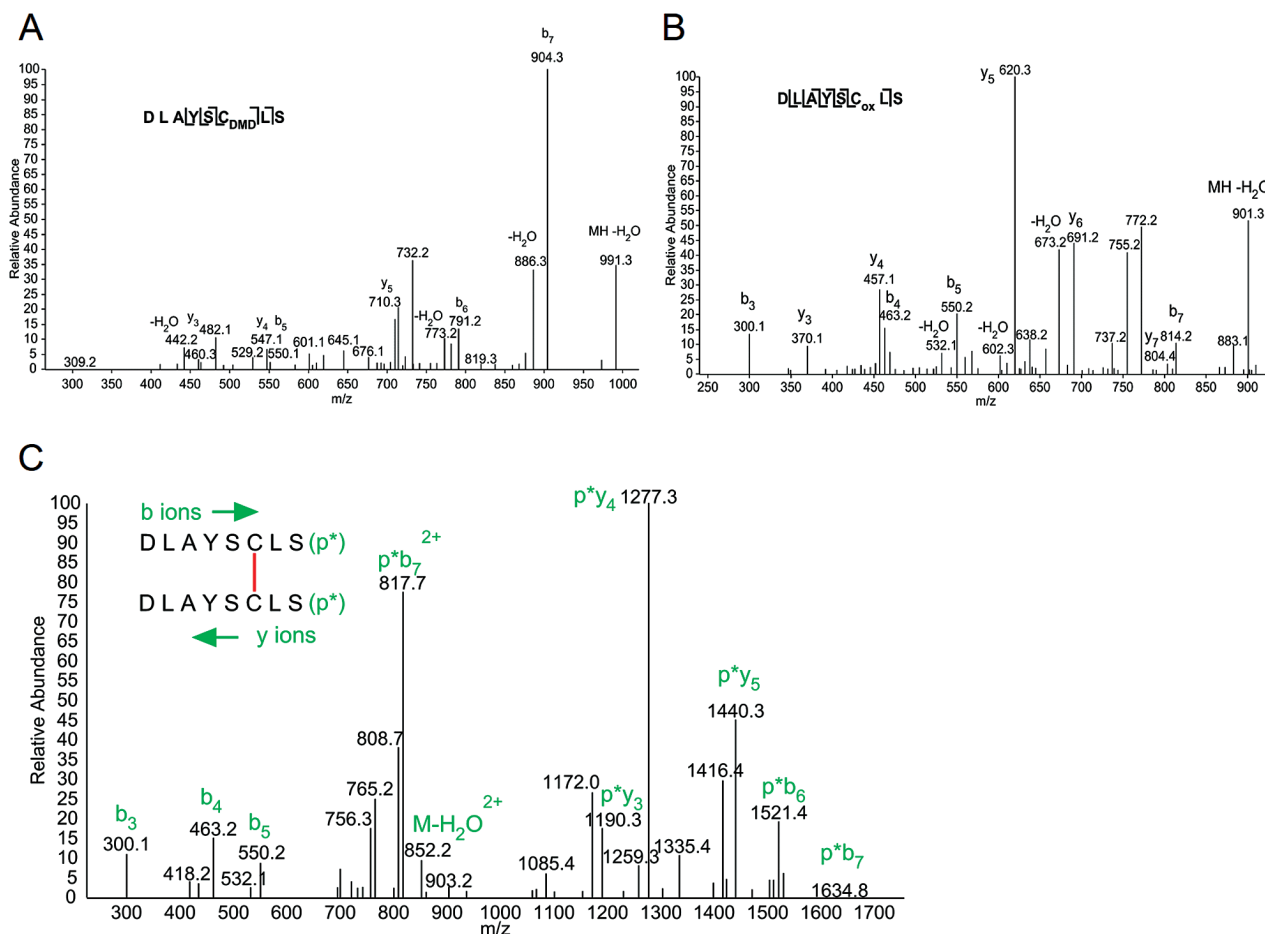


Fig. 2. Identification of oxidative cysteine modifications of cytMDH1_{Ox} by LC-MS/MS. (A) Sulfenylation identified on the Cys330-modified peptide. MS/MS spectrum of the singly charged parent ion $[M+H]^+ = 1009.3$. The y and b fragments indicate that Cys330 is present as a dimeric group-modified sulfonic acid (+138.0681 Da). (B) Sulfonic acid identified on the Cys330-modified peptide. MS/MS spectrum of the singly charged parent ion $[M+H]^+ = 919.3$. The y and b fragments indicate that Cys330 is present as a sulfonic acid (+48 Da). (C) Disulfide linked peptide identification by using Dbond software. A doubly charged parent ion of $[M+2H]^{2+} = 871.5$ Da shows fragmentation characteristics of a disulfide linkage between two Cys330s of MDH. The y and b series of ions allow the exact localization of the disulfide bridge, p* one strand of the dipeptide. (This figure is available in colour at JXB online.)

cat2 mutant when compared with wild-type Arabidopsis plants (Jacques *et al.*, 2015). All together, we showed that several Cys and Met residues of cytMDH1 are sensitive to oxidation.

Kinetics are affected by H₂O₂ and TRXh reactivates cytMDH1

We next investigated the effect of cytMDH1 oxidation on its enzymatic activity. The cytMDH1 activity progressively decreased upon H₂O₂ treatment. No decrease in activity could be observed for the DTT-treated sample over the same time range and temperature, indicating that oxidation of cytMDH1 is the major factor leading to loss of activity (Fig. 3A).

To understand how oxidation changes the activity of cytMDH1, we compared the kinetic parameters of cytMDH1_{Red} and cytMDH1_{Ox} (Fig. 3B, C; Table 1). The k_{cat} is 2-fold higher for cytMDH1_{Red} than that of cytMDH1_{Ox}, indicating a decrease of the rate of substrate conversion when the enzyme is oxidized. Interestingly, the K_M of cytMDH1_{Ox} for OAA is $143 \pm 15 \mu\text{M}$, which is 40% lower than that of 238

$\pm 21 \mu\text{M}$ for cytMDH1_{Red}. Similarly, the K_M of cytMDH1_{Ox} for NADH ($50 \pm 5 \mu\text{M}$) is also 30% lower than that for cytMDH1_{Red} ($72 \pm 7 \mu\text{M}$) (Fig. 3B, C; Table 1), indicating small increases in affinity for both substrates when cytMDH1 is oxidized. Overall, the k_{cat}/K_M values for OAA are similar for cytMDH1_{Red} and cytMDH1_{Ox}. The k_{cat}/K_M value of cytMDH1_{Ox} for NADH is $6.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, which is in the same range as the $9.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ value obtained for cytMDH1_{Red}. This suggests that a K_M adjustment occurs, to compensate for the k_{cat} decrease between cytMDH1_{Red} and cytMDH1_{Ox}.

Previously, it has been shown that TRXh1 reactivates cytMDH1_{Ox} by reducing a disulfide bond at the Cys330 residue (Hara *et al.*, 2006). The Arabidopsis genome encodes five cytosolic TRX isoforms (TRXh1–TRXh5) that might reduce cytMDH1. In order to test if TRXhs could reduce cytMDH1, we first tested protein interactions between recombinant TRXh1–TRXh5 and MDH isoforms in a yeast two-hybrid system (Vignols *et al.*, 2005). In order to stabilize redox-sensitive interactions, the resolving cysteine of the TRX active site was mutated to a serine. All five TRXh isoforms interacted with cytMDH1. However, no interaction

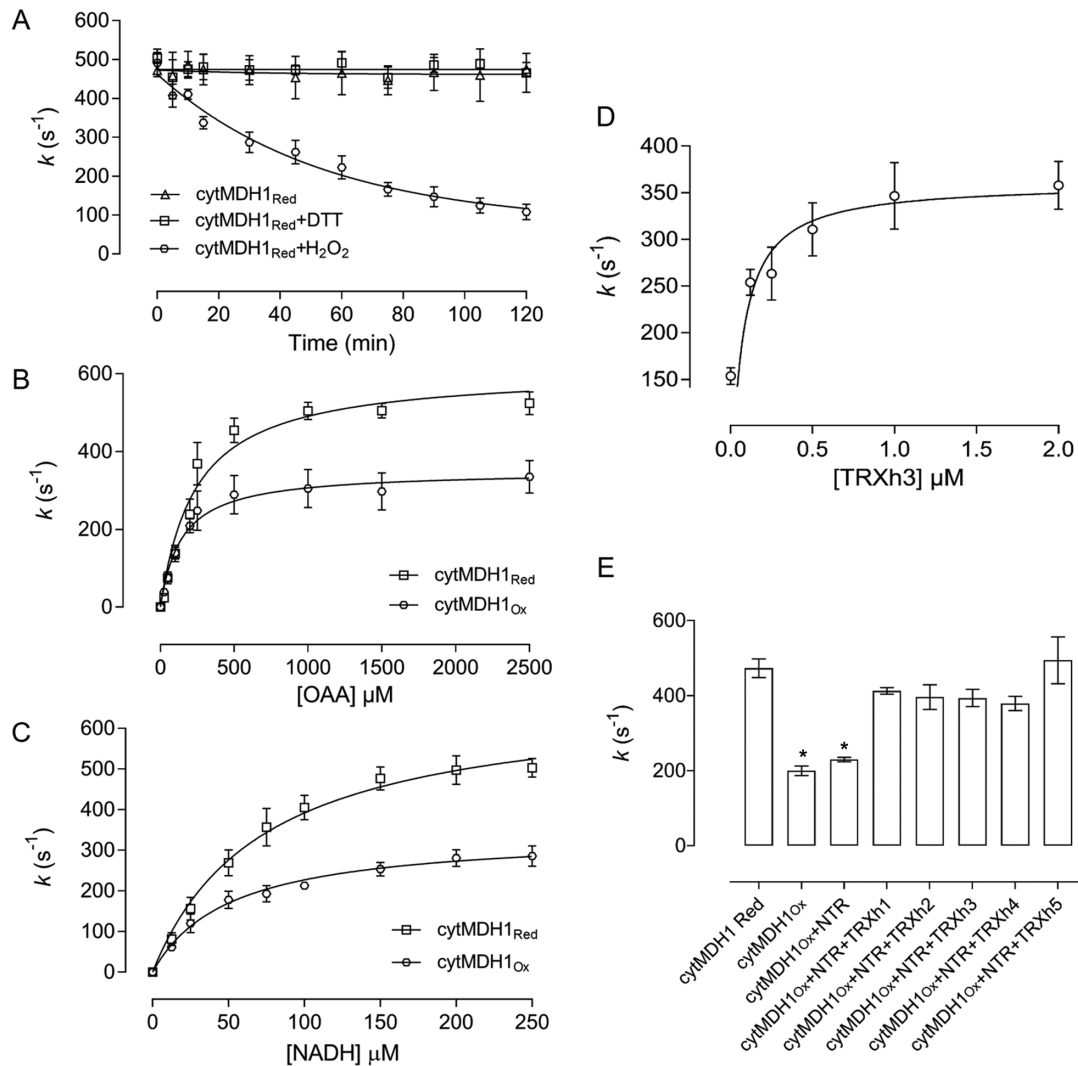


Fig. 3. CytMDH1 is sensitive to oxidation and reactivated by TRXh *in vitro*. (A) The time course of cytMDH1 activity was measured under reducing or oxidizing conditions. cytMDH1_{Red} at 8 μM was incubated with buffer (cytMDH1_{Red}), with 10 mM DTT (cytMDH1_{Red}+DTT), or with 10 mM H_2O_2 (cytMDH1_{Red}+ H_2O_2) at 30 °C. The plot of k (s^{-1}) versus time of treatment is shown. Data presented are means $\pm$ SD ($n > 3$). (B and C) CytMDH1 activity was measured at different concentrations of substrates: (B) OAA (0–2.5 mM) or (C) NADH (0–0.25 mM). The initial velocity (v_i) for each substrate concentration was measured, and the k (s^{-1}) values versus [OAA] or [NADH] were plotted and fitted with the Michaelis–Menten equation to obtain the kinetic parameters K_M and specific activity. At least three independent replicates of k were measured for each substrate concentration. (D) CytMDH1_{Ox} was incubated with increasing concentrations of recombinant TRXh3 in the presence of NADPH and NTRA. After 30 min incubation, the mix was diluted 40-fold and used for NAD-MDH activity assay. Data presented are means $\pm$ SD ($n > 3$). (E) Reduced or H_2O_2 -oxidized cytMDH1 was incubated with different TRXhs (2 μM) in the presence of NADPH and NTRA. After 30 min incubation, the mix was diluted 40-fold and used for NAD-MDH activity assay. Data presented are means $\pm$ SD ($n > 3$). An asterisk indicates values significantly different from cytMDH1_{Red} by the Student's t -test at 5% (* $P < 5 \times 10^{-3}$).

Table 1. Steady-state parameters for reduced and oxidized cytMDH1

	K_M OAA (μM)	K_M NADH (μM)	k_{cat} OAA (s^{-1})	k_{cat} NADH (s^{-1})	k_{cat}/K_M OAA ($\text{M}^{-1} \text{s}^{-1}$)	k_{cat}/K_M NADH ($\text{M}^{-1} \text{s}^{-1}$)
cytMDH1 _{Red}	238 $\pm$ 21	72 $\pm$ 7	608 $\pm$ 22	677 $\pm$ 24	2.6 $\times 10^6$	9.0 $\times 10^6$
cytMDH1 _{Ox}	143 $\pm$ 15	50 $\pm$ 5	351 $\pm$ 11	343 $\pm$ 12	2.5 $\times 10^6$	6.9 $\times 10^6$

between the cytosolic TRXhs and mitMDH1, chlMDH, and NADP-MDH was observed, indicating a specificity of the TRXhs to interact with cytMDH1 (Supplementary Fig. S4). To test if MDHs are able to interact with TRXs other than TRXh, we performed a yeast two-hybrid test using the

chloroplastic TRXf1 and f2 as bait. All tested MDHs (mitMDH1, chlMDH, cytMDH1, and NADP-MDH) were able to interact with TRXf1 and f2, suggesting that TRXfs have a larger interaction spectrum than TRXhs for MDH isoforms (Supplementary Fig. S5).

We further tested whether recombinant TRXhs are able to restore the activity of H_2O_2 -oxidized cytMDH1. All TRXh1–TRXh5 isoforms identified as interactors of cytMDH1 by yeast two-hybrid assay were able to restore the activity of cytMDH1_{ox} to a level similar to that of cytMDH1_{red} (Fig. 3D, E), implying that the TRXh–MDH interaction occurs in a redox-dependent manner.

The thermodynamic stability of cytMDH1 is affected by H_2O_2

In order to understand how H_2O_2 affects the activity of cytMDH1, the secondary structures of reduced and oxidized cytMDH1 were compared with far UV-CD (Fig. 4A). cytMDH1_{red} and cytMDH1_{ox} are characterized by a typical α -helical and β -sheet secondary structure with negative peaks around 210–220 nm and a positive peak around 190 nm. However, the spectrum of cytMDH1_{ox} shows a pronounced modification of the secondary structure, indicating a more disordered protein. We further determined the influence of oxidation on the protein stability by following the unfolding of oxidized and reduced cytMDH1 at 222 nm from 15 °C to 75 °C by CD. We obtained a melting temperature (T_m) of cytMDH1_{red} of

54.74 °C, while the T_m of cytMDH1_{ox} dropped by almost 5 °C to 49.88 °C (Fig. 4B), indicating that cytMDH1_{ox} is less stable than cytMDH1_{red}. Thermodynamic processes, such as the thermal unfolding of cytMDH1, are characterized by a variation in entropy (ΔS), enthalpy (ΔH), and Gibbs free energy (ΔG) (Murphy and Freire, 1992). Thermal unfolding parameters specify a higher need for energy in order to disrupt intramolecular interactions such as Van der Waals forces, hydrogen bonds, and ionic salt bridges for cytMDH1_{red}, based on the $\Delta\Delta H$ value of 8.6 kcal mol⁻¹ (Table 2). These interactions are well known as the main intramolecular forces that keep proteins structured, indicating that the amount of energy to unfold a protein is directly proportional to the interactions previously mentioned (O'Brien and Haq, 2004). The more flexible a protein is, the higher are the number of possible conformations. This effect is represented by conformational entropy (O'Brien and Haq, 2004). The $\Delta\Delta S$ value of 23 cal mol⁻¹ KJ⁻¹ suggests that cytMDH1_{red} undergoes a larger conformational change on the way to reach its unfolded conformation than its oxidized counterpart (Table 2). This result indicates that before thermal denaturation, cytMDH1_{red} is in a more restricted number of conformations maintained by a larger amount of interactions when compared with cytMDH1_{ox}.

Crystal structure of cytMDH1

In an effort to gain structural insights into the effects of oxidative stress on cytMDH1, the crystal structures of cytMDH1_{red} and H_2O_2 -soaked cytMDH1 were determined to a resolution of 1.8 Å and 1.35 Å, respectively (www.PDB.org, ID: 5NUF and 5NUE). CytMDH1 crystallizes as a homodimer with an interfacing area of 1817 Å² as determined by PISA analysis, and exhibited the structural fold common to the cytosolic MDH from porcine heart (Fig. 5) (Birktoft *et al.*, 1989). NAD⁺ was modeled into the available density in the $mF_o - DF_c$ omit map at the nucleotide-binding cleft of cytMDH1. A sulfate ion was found to occupy the malate/OAA-binding site proximal to the nicotinamide head of NAD⁺, binding with five water molecules and the side-chain groups of residues Asn132, Arg163, His188, and Ser243.

Superposition of the individual protomers of the cytMDH1 homodimer revealed significant differences in the conformation of a flexible loop of Pro92–Val102 at the opening to the substrate-binding site. In one conformation, this loop is in a 'closed' conformation, and co-ordinates a sulfate ion through the backbone amide and side-chain head of Arg99. The alternative 'open' conformation of this flexible loop region was found to be more disordered, with elevated B-factors and relatively poor coverage of electron density. No significant structural differences were observed between the structures of cytMDH1 and the H_2O_2 -soaked cytMDH1 crystal, both structures aligning with a root mean square deviation (RMSD) of 0.266 Å, and the largest structural deviations arising in the flexible loop region described above (Fig. 5A).

Evidence of partial sulfoxidation was identified on Met56 and Met97. Sulfoxidation of Met56 appears to have no direct effect on the local structural environment. The Met97 is located on the flexible loop proximal to the malate/

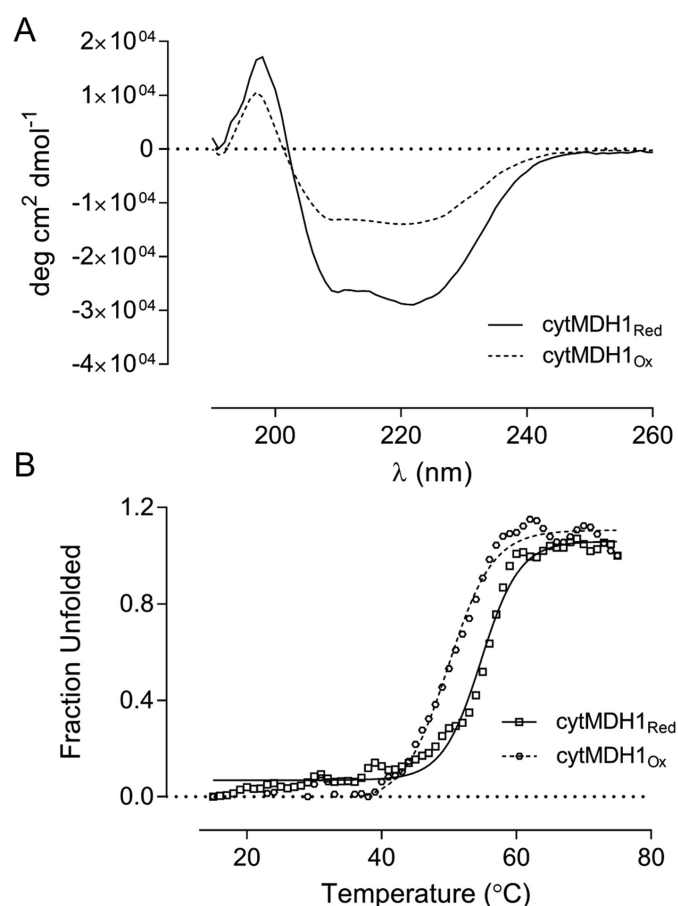


Fig. 4. CD of reduced and oxidized cytMDH1. (A) The far CD spectra for the cytMDH1_{red} and cytMDH1_{ox} were obtained with an average of three scans for each spectrum with a range from 190 nm to 260 nm. (B) Thermal unfolding monitored at 222 nm was carried out for cytMDH1_{red} and cytMDH1_{ox} by increasing the temperature from 15 °C to 75 °C.

Table 2. T_m and thermodynamic parameters of reduced and oxidized cytMDH1 thermal denaturation

	ΔH (kcal mol ⁻¹)	ΔS (cal mol ⁻¹ KJ ⁻¹)	ΔG (kcal mol ⁻¹)	$\Delta\Delta H^a$ (kcal mol ⁻¹)	$\Delta\Delta S^a$ (cal mol ⁻¹ KJ ⁻¹)	$\Delta\Delta G^a$ (kcal mol ⁻¹)	T_m (°C)
cytMDH1 _{Red}	63.4 ± 0.2	192 ± 1	5.10 ± 0.01	8.6	23	1.5	54.7 ± 0.2
cytMDH1 _{Ox}	54.8 ± 0.6	169 ± 1	3.62 ± 0.02				49.8 ± 0.1

$\Delta\Delta$ stands for reduced minus oxidized.

^a Thermodynamic constants calculated at 30 °C.

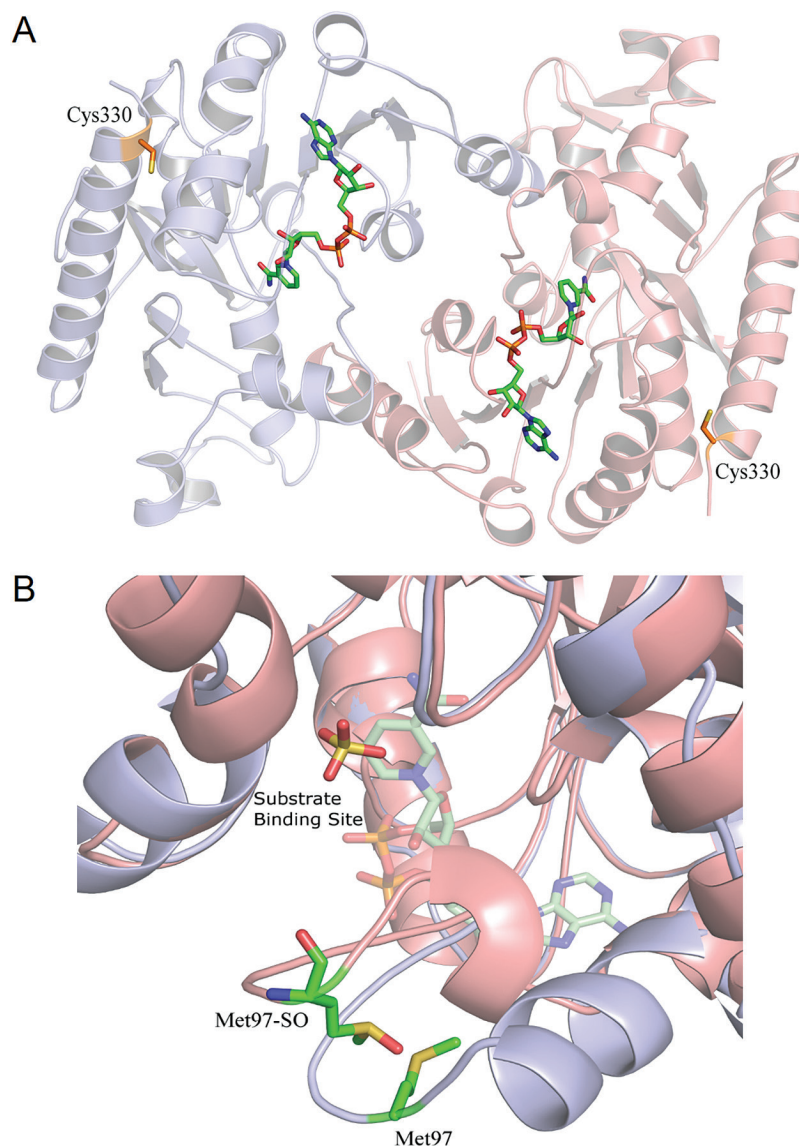


Fig. 5. Crystal structures of cytMDH1 and sulfoxidation. (A) The conserved dimeric structural arrangement of MDH places the C-termini at opposite ends. Here in the crystal structure of Arabidopsis cytMDH1, each protomer is colored separately, and the side chain of Cys330 and NAD⁺ are colored orange and green, respectively. (B) Met97 is located on a flexible loop proximal to the OAA/malate-binding site, which in the crystal structure is occupied by a sulfate ion. Superposition of two subunits of the cytMDH1 homodimer shows that this flexible loop can be in either a closed (pink) or an open (blue) conformation independent of the loop conformation of the neighboring dimer subunit. Sulfoxidation of Met97 observed in the crystal structure of cytMDH1 is indicated, though it should be noted that this flexible loop region can adopt a closed/open conformation irrespective of Met97 sulfoxidation.

OAA-binding site, which has also been previously identified to form methionine sulfoxide in Arabidopsis plants (Jacques *et al.*, 2015). Sulfoxidation of Met97 could conceivably affect mobility of the Pro92–Val102 loop region, and thereby possibly influence substrate delivery to the active site (Fig. 5B). However, we found no significant difference in activity after

we oxidized the IAM-pretreated cytMDH1 with 10 mM H₂O₂ (Supplementary Fig. S6), indicating that methionine oxidation does not play a major role in the effect of oxidation on the enzymatic capacity of cytMDH1.

From a $mF_o - DF_c$ difference map, additional positive density adjoining the sulfur of Cys330 was observed in two

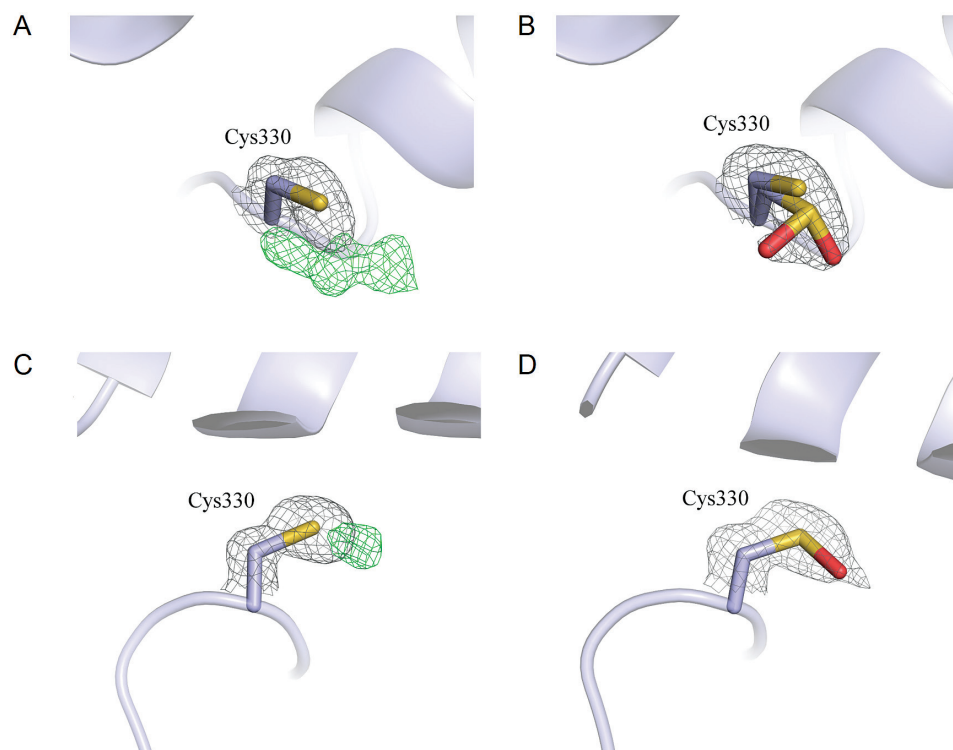


Fig. 6. Cysteine oxidation observed in the crystal structure of H_2O_2 -treated cytMDH1. The presence of additional density arising in the F_o-F_c map at Cys330 of chain B (A) and chain C (C) prompted modelling of a partial-occupancy sulfenic and sulfonic acid (B and D, respectively). Shown are $2mF_o-DF_c$ (gray) and mF_o-DF_c (green) electron density maps contoured at $0.33 \text{ e } \text{\AA}^{-3}$ and $0.34 \text{ e } \text{\AA}^{-3}$, respectively, for (C), and $0.41 \text{ e } \text{\AA}^{-3}$ and $0.295 \text{ e } \text{\AA}^{-3}$ for both (B) and (D). The $2mF_o-DF_c$ map for (A) is contoured at $0.25 \text{ e } \text{\AA}^{-3}$.

of the three molecules of the asymmetric unit of cytMDH1. Into this extra density, partial-occupancy sulfenylation or sulfinylation at Cys330 was modeled for the respective molecules of cytMDH1 (Fig. 6). The partial occupancy of the cysteine sulfenylation and sulfinylation may be due to the short duration of the H_2O_2 crystal soak, resulting in an unequal distribution of peroxide throughout the crystal unit cells.

Redox sensitivity of NAD-MDHs varies in different plant cell compartments

To obtain an overview of the redox sensitivity of NAD-MDH in the plant cell, we measured the NAD-MDH activity in purified cytosol, mitochondrion, and chloroplast fractions before and after the 10 mM H_2O_2 treatment. Both the cytosolic and chloroplastic NAD-MDH activities were significantly decreased after the H_2O_2 treatment (40% and 20%, respectively) (Fig. 7A). No significant difference of the mitochondrial NAD-MDH activities between the non-treated and treated samples was observed.

In order to study the impact of H_2O_2 on the MDH gene expression, we analyzed the microarray and RNA-Seq data for all MDH genes (Supplementary Fig. S7). Microarray analyses were performed on 7-day-old wild-type (Col-0) Arabidopsis seedlings without and with 10 mM H_2O_2 treatment for 24 h (Willems *et al.*, 2016). RNA-Seq analysis was done on 21-day-old Col-0 and *cat2* mutant Arabidopsis seedlings under photorespiratory stress (Waszczak *et al.*, 2016).

No major change was found for mRNA levels of all MDHs upon stresses from both data sets. From the RNA-Seq data set, similar mRNA levels of MDHs were found between the Col-0 wild-type and *cat2* mutant Arabidopsis plants. Collectively, these data indicate that H_2O_2 has little influence on the transcriptional level of MDHs, and that the impact of H_2O_2 on NAD-MDH activities is probably due to post-translational modifications.

Mutations in *cat2* and *ntra ntrb* lower the *in vivo* NAD-MDH activity

We further evaluated the NAD-MDH activity in a *cat2* loss-of-function mutant, in which the major catalase isoform in leaves is inactivated, retaining only 8% of extractable catalase activity (Mhamdi *et al.*, 2010b) (Supplementary Fig. S8). The NAD-MDH activity was significantly decreased in the *cat2* mutant when compared with wild-type plants (25–33%), indicating that the NAD-MDH activity is affected by increased *in vivo* H_2O_2 levels (Fig. 7B).

As we showed that the reduction in cytMDH1_{ox} depends on cytosolic TRXh, we also measured the NAD-MDH activity in the TRX reductase double mutant *ntra ntrb* in which TRXh are partially inactive (Reichheld *et al.*, 2007). As previously observed (Daloso *et al.*, 2015), we also found a significant decrease (17–20%) in this mutant. However, decreasing the *NTR* gene expression with RNAi in *cat2* (*cat2 ntra ntrb*) had no additional impact on the NAD-MDH activity, suggesting that the residual NTR activity in the RNAi lines

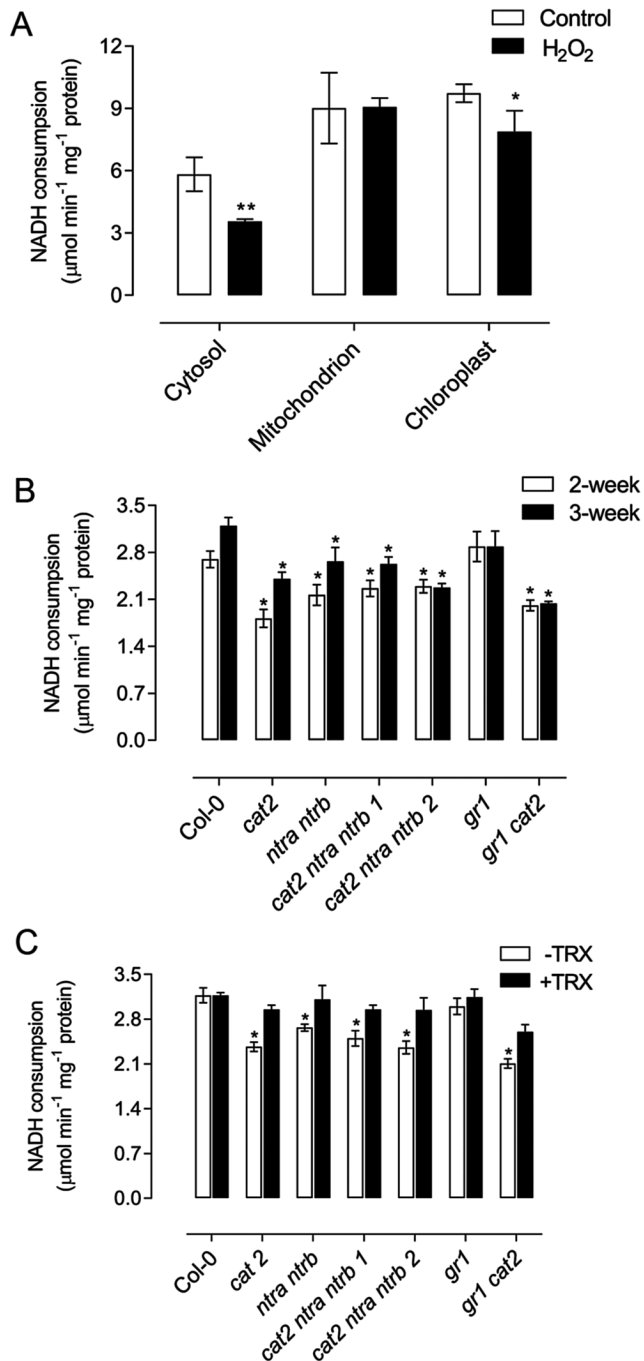


Fig. 7. NAD-MDH activity in different cell compartments and mutants. (A) The NAD-MDH activity was measured for different cell compartments in wild-type (Col-0) plant extracts. A 3 μg aliquot of protein extracts was used for activity assays. Data presented are means ±SD ($n \geq 3$). An asterisk indicates values significantly different from the wild type by the Student's t -test (* $P < 0.05$, ** $P < 0.01$). (B) The NAD-MDH activity was measured in wild-type (Col-0) and different mutant plants grown in soil for 2 weeks (white bars) or 3 weeks (black bars). A 5 μg aliquot of protein extracts was used for activity assays. (C) Protein-based complementation assays were performed in the same mutants as in (A). A 5 μg aliquot of protein extracts was untreated (black bars) or incubated in 1 mM NADPH in the presence of 4.59 μM TRXh3 and 3.12 μM NTRA for 2 h on ice (white bars). This reaction mixture was diluted 40 times before performing the NAD-MDH activity assay. Data presented are means ±SD ($n = 4$). An asterisk indicates values significantly different from the wild type by the Student's t -test at 5% (* $P < 5 \times 10^{-3}$).

is efficient enough to reduce the oxidized MDH (Fig. 7B; Supplementary Figs S8, S9).

We further tested whether the glutathione-reducing system could be part of the cytMDH redox regulation mechanism. Therefore, the NAD-MDH activity was measured in a cytosolic glutathione reductase mutant (*gr1*) with a more oxidized glutathione redox state (Supplementary Table S2). No decrease in NAD-MDH activity was observed, and in the *gr1 cat2* double mutant, the decrease in NAD-MDH activity was similar to that observed in the *cat2* mutant.

Finally, to confirm that the decreased NAD-MDH activities in *cat2* and the *ntra ntrb* double mutants are linked to TRX reduction, we performed NAD-MDH activity assays for the plant extracts in the presence of recombinant cytosolic NTRA and TRXh3 proteins (Fig. 7C). After the addition of the NTR/TRXh3 system in the wild-type plant extracts, the NAD-MDH activity remained the same. However, the activity was significantly increased in the plant extracts from *cat2* and *ntra ntrb* mutants, confirming a TRX-dependent activity of the MDH pool.

Discussion

Plant NAD(P)-dependent MDHs are part of a multigenic family (Supplementary Fig. S1). The redox regulation of chloroplastic NADP-MDH has been extensively studied in the last decades. However, a redox regulation of other isoforms of NAD-MDH is unlikely to act in a similar way. Accordingly, the mitochondrial NAD-MDH isoforms that are involved in the tricarboxylic acid (TCA) cycle are not prone to redox regulation (Yoshida and Hisabori, 2016), though other enzymes of the TCA cycle have been shown to be redox regulated (Daloso *et al.*, 2015). Our NAD-MDH activity assays performed on purified subcellular fractions also support redox-independent mitochondrial NAD-MDH activities. However, the H₂O₂ sensitivity of the chloroplastic NAD-MDH activity might indicate a redox dependence of chloroplastic NAD-MDH *in vivo*, which was not previously found *in vitro* (Berkemeyer *et al.*, 1998). A potential redox regulation of the chloroplastic NAD-MDH activity will need to be further explored.

In clear contrast to mitochondrial isoforms, different arguments are in favor of a redox regulation of the Arabidopsis cytMDH1 (Hara *et al.*, 2006; Hara and Hisabori, 2013; this work): (i) cytMDH1 was identified as a sulfenylated protein under oxidative stress and during symbiosis (Waszczak *et al.*, 2014; Akter *et al.*, 2015), which has been validated *in vitro* in our study (Fig. 1; Supplementary Fig. S10); (ii) not only cysteine sulfenylation, but also cysteine overoxidation and methionine sulfoxidations occur under oxidizing conditions (Figs 3, 6, 7; Supplementary Table S2); (iii) modifications in enzymatic kinetics, secondary structure, and folding characteristics are observed under H₂O₂ treatment (Figs 3–5); and (iv) NAD-MDH activities are affected in H₂O₂-supplied purified cytosolic fractions, and in mutants accumulating high levels of

H₂O₂ (*cat2*) or impaired in the TRX reduction systems (*ntra ntrb*) (Fig. 7). Therefore, our present work extends *in vitro* data demonstrating a redox regulation of cytMDH1, and we provide clear evidence that this regulation occurs *in vivo*.

All eukaryotic cytosolic NAD-MDHs exist as homodimers (Berkemeyer *et al.*, 1998; Birktoft *et al.*, 1989), which was confirmed for the cytMDH1 isoform by SEC (Hara *et al.*, 2006). Our structural data also confirm this observation (Fig. 6). Moreover, we show that a major conformational change occurs between cytMDH1_{Red} and dimeric cytMDH1_{Ox}. Whereas the cytMDH1_{Red} dimer is mainly stabilized by electrostatic and hydrophobic interactions, as visualized by SEC and native gels (Supplementary Figs S2, S3), oxidation causes the formation of a disulfide-linked dimer (Figs 2, 3). It has been hypothesized that an intermolecular Cys330–Cys330 bond co-ordinates a tetrameric structure (Hara *et al.*, 2006), but not supported by our SEC and native gel profiles, because no tetrameric structure was observed (Supplementary Figs S2, S3), possibly due to different oxidation conditions between the two studies: we used H₂O₂-induced oxidation, while CuCl₂-induced oxidation was used in the previous study (Hara *et al.*, 2006).

The H₂O₂-mediated cytMDH1 oxidation inducing a conformational modification is reminiscent of the conformational change reported for oxidized chloroplastic NADP-MDH (Ruelland *et al.*, 1998). The formation of the C-terminal intramolecular disulfide bond upon oxidation of this enzyme triggers the binding of the C-terminal domain to OAA. In contrast, cytMDH1 oxidation has a minor effect on the affinity for the substrate, even slightly decreasing the *K_M* for OAA and NADH. Therefore, a similar redox regulatory mechanism between the two isoforms can be ruled out. However, the observed decrease in the turnover rate (*k_{cat}*) of the cytMDH1_{Ox} suggests that this modification of the conformation affects the catalytic activity of the enzyme. Surprisingly, the comparison of the crystal structures of reduced and oxidized cytMDH1 does not show extensive changes in the global conformational fold of both structures. However, we should note that the crystal structure of oxidized cytMDH1 is a crystal of cytMDH1_{Red} incubated with H₂O₂. Therefore, the constraints of the crystal lattice preclude the possibility of large-scale conformational changes. As both cytMDH1_{Red} and cytMDH1_{Ox} form homodimers, it is difficult to separate cytMDH1_{Ox} from the cytMDH1_{Red} for cytMDH1_{Ox} crystallization trials. Although Met97 at the substrate-binding site was sensitive to oxidation (Fig. 6), the enzyme kinetics show no major impact of oxidation on the affinity of cytMDH1 towards OAA and NADH (Table 1). The activity of alkylated cytMDH1 is not sensitive to H₂O₂ oxidation, whereas cytMDH1_{Red} is very sensitive to diamide (Supplementary Fig. S6), a reagent that introduces disulfide bonds. Altogether, our results indicate that the decrease in activity is not due to methionine oxidation, but rather to disulfide bond formation.

Among the cysteine residues of cytMDH1 undergoing oxidative modifications, Cys79 is prone to overoxidation, while Cys330 tends preferentially to form a Cys330–Cys330 disulfide bond (Supplementary Table S2), suggesting that disulfide formation protects Cys330 from overoxidation (Fig. 7).

We also demonstrated that sulfenylation of Cys does not by itself impact the activity of cytMDH1 (Supplementary Fig. S10). Therefore, we conclude that in contrast to another sulfenylated residue, Cys79, the sulfenylated Cys330 forms a disulfide bond with free Cys330 thiol (Fig. 8), which causes the reversible conformational change and a decrease of the activity.

Intriguingly, our crystal structure data show that Cys330 residues from two interacting subunits are located at opposite sides in the cytMDH1 dimeric molecule (Fig. 6). A hypothesis is that upon H₂O₂ oxidation, a major conformational change brings Cys330 of both subunits in close proximity to permit Cys330–Cys330 disulfide bond formation. As mentioned above, our oxidized crystal structure does not allow the visualization of such conformational changes and we would need to obtain a crystal structure after oxidation of the protein in solution, but have failed as yet. Another more likely hypothesis is that the disulfide bond is formed between Cys330 of two individual non-covalent dimers. As a tetrameric cytMDH1 state was not detected, we suggest that Cys330–Cys330 formation between dimers leads to structural changes that disrupt the native non-covalent dimer, leading to a population of covalent homodimers (Fig. 8). This scenario is supported by the CD analyses demonstrating that cytMDH1_{Ox} is significantly more disordered (Fig. 4A) than the cytMDH1_{Red}, comprising a larger number of possible states prior to thermal denaturation (Fig. 4B; Table 2), and that cytMDH1_{Red} is in a more restricted number of possible conformations. Additionally, $\Delta\Delta G$ and *T_m* values also indicate that cytMDH1_{Red} is significantly more stable than cytMDH1_{Ox} (Table 2). CD and steady-state kinetic data reinforce the final conclusion that oxidation of cytMDH1 induces a conformational disorder and increases instability of the protein, possibly caused both by Cys330–Cys330 disulfide formation and overoxidation. Furthermore, the disulfide bond dimer can be efficiently reactivated by TRXh proteins, providing full reversibility to the system and further demonstrating the role of the disulfide bond in protecting the enzyme activity (Fig. 8).

Interestingly, the Cys330 is also conserved in the other two cytMDH isoforms encoded by the Arabidopsis genome (Supplementary Fig. S1) as well as in cytMDH orthologs of other plants, suggesting that this mechanism is conserved in the green lineage. It is also likely that the decrease in NAD-MDH activities observed in the *cat2* mutant is reporting the inactivation of all three cytosolic isoforms. Altered activity is also found in the *ntra ntrb* mutants, where the TRX system is partially inactivated (Reichheld *et al.*, 2007). Decreased NAD-MDH activity is probably due to the lower capacity of the *ntra ntrb* mutant to regenerate oxidized MDH *in vivo*. The Arabidopsis genome harbors multiple cytosolic TRXs (Meyer *et al.*, 2012). We have tested five isoforms of TRXh with cytMDH1 as substrate, and all of them are able to restore the cytMDH1 activity after oxidation. It is likely that these TRX isoforms are redundant, which also explain the decreased NAD-MDH activity that is only observed in the *ntra ntrb* mutant, and not with several TRXh single mutants (*trxh1*, *trxh2*, *trxh3*, *trxh4*, and *trxh5*) tested so far (data not shown). Interestingly, our yeast two-hybrid test showed that cytosolic

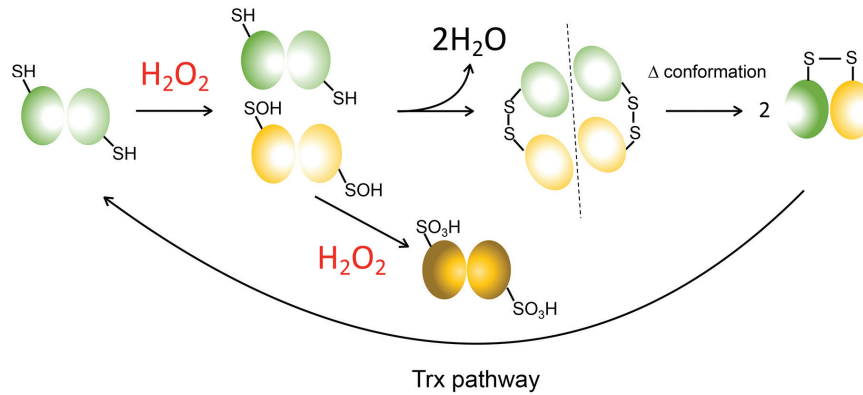


Fig. 8. How cytMDH1 copes with H_2O_2 oxidation. Oxidation of homodimeric cytMDH1 leads to sulfenylation of Cys330 and the formation of an intermolecular Cys330–Cys330 disulfide. Therefore, a major conformational change is required that leads to the formation of a thermodynamically less stable homodimer with an altered kinetic behavior but with similar $k_{\text{cat}}/K_{\text{M}}$ values. The S–S linked dimer protects the Cys330 from irreversible overoxidation and becomes a substrate for the TRX pathway. Methionine sulfoxidation and sulfenylation of cysteines other than Cys330 do not affect the kinetic behavior.

TRXhs interact specifically with cytMDH1. As Cys330 is only found in cytosolic MDHs, it is tempting to speculate that TRXh/cytMDH forms a mixed disulfide between the catalytic Cys of TRXh and Cys330 of cytMDH1, although other structural features could mediate this specific interaction.

Cytosolic MDH plays a key role in equilibrating reducing equivalents between the organelles as well as in providing an important pool of OAA for further metabolic activities. Therefore, its activity needs to be preserved under stress conditions leading to reduced availability of its substrates. Our data clearly suggest that the redox regulation of cytMDH is playing an efficient protective role to avoid irreversible overoxidation of the enzyme. Interestingly, our kinetic data show that the cytMDH1_{ox} catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) is not profoundly perturbed compared with that of cytMDH1_{red}. While the turnover rate (k_{cat}) is 2-fold decreased, the affinity (K_{M}) for the substrates is slightly increased, suggesting that cytMDH1_{ox} is adapting its activity to low availability of the substrate. Such a buffering mechanism as well as the reversibility of the regulation by the TRX system probably acts as a safety mechanism to minimize oxidative inactivation of cytMDH1 under stress conditions. Such a fine-tuned redox regulation is also consistent with the fact that the cytMDH1 protein is abundantly maintained under oxidative stress, in order to adapt the cell metabolism under changing environmental conditions.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Alignment of *Arabidopsis thaliana* MDHs.

Fig. S2. Dimeric forms of reduced and oxidized cytMDH1 identified by size exclusion chromatography.

Fig. S3. Reduced and oxidized cytMDH1 in native gel.

Fig. S4. Cytosolic TRXhs interact with cytMDH1.

Fig. S5. Plastidial TRXf1 and TRXf2 interact with members of the MDH family.

Fig. S6. Redox sensitivity of cytMDH1 is regulated by TRXh on the disulfide bridge.

Fig. S7. Transcriptional analysis of MDHs under stress conditions.

Fig. S8. Catalase activity in mutants.

Fig. S9. NTR accumulation is decreased in RNAi lines.

Fig. S10. Sulfenylation does not affect the activity of cytMDH1.

Table S1. Mass spectral summary of post-translational oxidation modifications on cysteines and methionines.

Table S2. Glutathione levels in the *gr1* mutant.

Table S3. Crystallographic data collection and refinement statistics

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