Oxidation−Reduction Properties of the Regulatory Disulfides of Sorghum Chloroplast Nicotinamide Adenine Dinucleotide Phosphate−Malate Dehydrogenase[†]

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ABSTRACT: Oxidation−reduction midpoint potentials (E_m) have been measured for the thioredoxin-dependent, reductive activation of sorghum nicotinamide adenine dinucleotide phosphate- (NADP-) dependent malate dehydrogenase (MDH) in the wild-type enzyme and in a number of site-specific mutants. The E_m value associated with activation of the wild-type enzyme, −330 mV at pH 7.0, can be attributed to the E_m of the C365/C377 disulfide present in the C-terminal region of the enzyme. The C24/C29 disulfide, located in the N-terminal region of the enzyme and the only other disulfide present in oxidized, wild-type MDH, has a E_m value of −280 mV at pH 7.0. A third regulatory disulfide, C24/C207, that is absent in the oxidized enzyme but is thought to be formed during the activation process, has an E_m value at pH 7.0 of −310 mV. E_m vs pH profiles suggest pK_a values for the more acidic cysteine involved in the formation of each of these disulfides of 8.5 for C24/C29; 8.1 for C24/C207; and 8.7 for C365/C377. The results of this study show that the N-terminal disulfide formed between C24 and C29 has a more positive E_m value than the two other disulfides and is thus is likely to be the "preregulatory disulfide" postulated to function in activating the enzyme.

The chloroplast-located nicotinamide adenine dinucleotide phosphate−malate dehydrogenase (NADP-MDH,[1] E.C. 1.1.1.82) plays a crucial role in C_4 plants, where malate serves as the mobile form of reducing power that shuttles between the bundle sheath and mesophyll cells (*1*). While the physiological importance of NADP-MDH is less obvious in C_3 plants, it may serve as part of a "malate valve" that functions under conditions of excess reducing power (*2*). Chloroplast NADP-MDH enzymes from both C_3 and C_4 plants share a common regulatory mechanism, involving the reduction of regulatory disulfides in the enzyme (*1*). The regulation in vivo is thought to occur through thiol−disulfide interchange with reduced thioredoxin (*1*). Thioredoxin is reduced, in a reaction catalyzed by ferredoxin:thioredoxin reductase, by the reduced ferredoxin produced by the photochemical reaction of photosystem I (*1*). Of all the chloroplast enzymes regulated by the ferredoxin−thioredoxin system, NADP-MDH is the most redox-dependent, having no detectable activity in the oxidized form (*1*). It also displays the most complex activation pattern seen for a thioredoxin-regulated chloroplast enzyme, with the net reduction of two disulfides (involving an additional dithiol/disulfide exchange as an intermediate step) per monomer required for the enzyme to reach full activity (*1, 3*). The overall activation process is relatively slow, requiring approximately 10 min in vitro, and displays complex kinetics that include a distinct lag phase (*1*). Both of the chloroplast thioredoxins, thioredoxin *f* and thioredoxin *m*, are able to serve as effective reductants in the activation of MDH (*1*). In contrast, nonphysiological reductants for disulfides, such as dithiothreitol (DTT), are unable to activate the enzyme (*1*).

The observation that a pretreatment with mercaptoethanol did not activate NADP-MDH, but eliminated the activation lag phase upon subsequent addition of reduced thioredoxin led Hatch and Agostino (*4*) to propose that the reduction of a "preregulatory" NADP-MDH disulfide was necessary before the reduction of a "regulatory" disulfide could take place. Independent evidence for the participation of two disulfides in the activation of NADP-MDH, one located in the amino-terminal portion of the enzyme and one located in the carboxy-terminal portion, was provided by a series of chemical modification and site-directed mutagenesis studies on the sorghum chloroplast enzyme (*1*). The N-terminal regulatory disulfide of sorghum NADP-MDH was identified as involving cysteines 24 and 29 and the C-terminal regulatory disulfide was shown to link cysteines 365 and 377 (*1*). The presence of these C24/C29 and C365/C377 disulfides as the only two disulfides in the oxidized, inactive form of the sorghum enzyme has recently been confirmed

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[1] Abbreviations: Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DTT, dithiothreitol; MDH, malate dehydrogenase; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NADP, nicotinamide adenine dinucleotide phosphate; Tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane.

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from the X-ray crystal structure of the protein (*5*). The X-ray crystal structure of the thioredoxin-regulated chloroplast NADP-MDH from *Flaveria bidentis* also reveals the presence of one disulfide in the N-terminal region of the enzyme and one disulfide in the C-terminal region (*6*). Subsequent studies revealed that a fifth cysteine residue, C207, was also involved in the regulation of sorghum NADP-MDH (*3, 7*). A mechanism was proposed in which the initial reaction with thioredoxin leads to replacement of the original C24/C29 disulfide by a new C24/C207 disulfide that must be reduced, along with the C365/C377 disulfide, to produce the fully activated sorghum enzyme (*3, 7*). Recent evidence suggests that a transient mixed disulfide between C207 of the sorghum enzyme and thioredoxin may be formed during the activation of the enzyme (*7*).

Hatch and Agostino (*4*) had proposed that the preregulatory disulfide had a more positive oxidation−reduction midpoint potential (E_m) value than did the regulatory disulfide. An E_m value of −330 mV was reported for the activation of maize NADP-MDH at pH 7.0 (*8*), and an E_m value of −370 mV for activation of spinach NADP-MDH at pH 8.0 can be calculated from the equilibrium constant reported for the oxidation−reduction reaction between DTT and the enzyme at this pH (*9*; R. Scheibe, personal communication). However, the oxidation−reduction properties of the different individual disulfides identified in NADP-MDH activation have never been determined. The availability of site-directed mutants of NADP-MDH (*3, 10, 11*), each of which contains only one regulatory disulfide, provides the opportunity to determine the oxidation−reduction potentials of each of the disulfides separately. The results of this study, reported below, show that the N-terminal disulfide formed between C24 and C29, has a more positive E_m value than the two other disulfides and is thus a good candidate for being the "preregulatory disulfide" postulated by Hatch and Agostino (*4*).

MATERIALS AND METHODS

cDNAs encoding sorghum NADP-MDH (*12*) were previously mutated at selected cysteines by the site-directed mutagenesis method of Kunkel (*13*). Mutants C207A (both the N-terminal C24/C29 and C-terminal C365/C377 disulfides present but without any possibility for the former to isomerize to a C24/C207 disulfide), C24S/C29S (C-terminal bridge C365/C377 remaining), ΔN (33 amino acids deleted at the N-terminus extension, C365/C377 disulfide remaining), C29S/C365A/C377A (C24−C207 disulfide present), C207A/C365A/C377A (N-terminal C24−C29 disulfide remaining), and C29S/C207A/C365A/C377A (a permanently active enzyme containing no disulfide) were prepared as described previously (*3, 10, 11*). A partially active MDH mutant, which contains the C-terminal C365/C377 disulfide but cannot form a disulfide involving either N-terminal cysteine and which has the penultimate negative charge removed (C29S/C207A/E387Q), was engineered as described previously (*14*). Figure 1 summarizes the status of the regulatory cysteines in these mutants. The mutated cDNAs were cloned in the expression vector peT 3d (*15*) and expressed in *Escherichia coli* strain BL21, and the resulting MDH proteins purified by previously described techniques (*3, 10, 11*).

Oxidation−reduction equilibration was carried out essentially as described by Rebeillé and Hatch (*8*), using

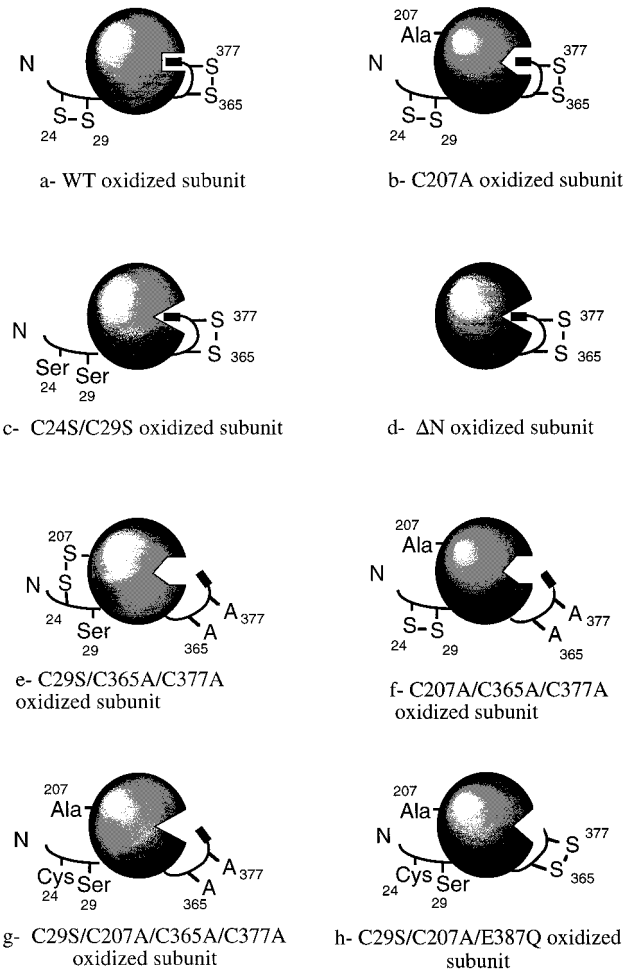

FIGURE 1: Schematic representation of the location of the regulatory disulfides, of the mutated cysteines in the various mutants of NADP-MDH, and of the consequences of the mutations on the conformation and accessibility of the active site. Only one monomer is represented for clarity. The N-terminus is on the left, and the C-terminus is on the right. The highly active conformation of the active site is represented by a triangular opening, the inactive conformation by a square, and a partially active conformation by an arrowhead-like opening. The black rectangular block at the C-terminus represents the negative charge of the penultimate glutamate, responsible for the anchoring of the C-terminal extension in the active site. This charge is removed in mutant C29S/C207A/E387Q (h). Whenever the active site is blocked by the C-terminus (a−d), the oxidized enzyme has no activity, even when its active site is in a highly active conformation (d, e). In panels a, c, and d, C207 is present but does not belong to a disulfide in the oxidized subunit; hence it is not represented.

mixtures of oxidized and reduced DTT to define the ambient redox potential (E_h) and *Escherichia coli* thioredoxin (10 μg in the 50 μL equilibration mixture) to equilibrate the enzyme with the DTT redox buffer. NADP-MDH (2 μg) was present in the equilibration mixture, along with DTT, at a total concentration of 10 mM. Buffers used, all at a concentration of 100 mM, were as follows: pH 5.5−6.7, MES; pH 6.7−7.5, MOPS; pH 7.4−8.8, Tricine; pH 8.5−9.5, Bis-Tris propane; and pH 9.5−10.0, CAPS. Redox equilibration was carried out for 10 min for the wild-type enzyme and 3 min for the mutants. E_m values were shown to be independent of total DTT concentration and time in the range around these values, as required for an equilibrium measurement. As an additional control, MDH activity was monitored after redox equilibration times of 30 min and it was found that no

additional increase in activity was observed with these longer times. Aliquots (10 μL) of the redox-equilibrated enzyme were assayed at 30 °C by following, for a total of 3 min, the decrease in absorbance at 340 nm corresponding to the oxidation of NADPH by oxaloacetate. The 1 mL reaction mixture for the activity assay contained 100 mM Tris buffer (pH 8.0), 140 μM NADPH, and 780 μM oxaloacetate. One unit of activity is defined as 1 μmol of NADPH oxidized min^{-1} (mg of enzyme)$^{-1}$.

Redox equilibrations for all of the titrations presented below were carried out without any precautions taken to remove oxygen, because of the insensitivity of reduced DTT to oxidation by oxygen (*17*). As a control, several titrations were also carried out under an argon atmosphere, using oxygen-free buffers and the system described by Hutchison and Ort (*17*), for wild-type MDH and its ΔN, C207A and C29S/C365A/C377A mutants. In all cases, identical E_m values were obtained in the presence and absence of oxygen. As a further control, the stability of the reduced DTT in the solutions used for redox buffering (prepared without any precautions to remove dissolved oxygen) was checked by titrating aliquots with the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) at 30-min intervals for times up to 3 h. No decrease in the thiol content of the DTT solutions was detected over this time period.

Best-fit values for E_m were determined as described previously (*16*). In the cases where multiple titrations were carried out for the same protein at a given pH, average values are given and the average deviation is reported to provide an estimate of the experimental uncertainties. All E_m value calculations were based on a value of −330 mV for the E_m of DTT at pH = 7.0. This value is an average of several closely agreeing values available in the literature (*17*). The value used for the pH dependence of the E_m value of DTT was −59 mV/pH unit over the pH range from 5.5 to 8.2 (*18*). Values of 9.2 and 10.1, respectively, were used for the pK_as of the more acidic and less acidic thiol groups of DTT (*18*) for calculating the E_m value of DTT at pH values above 8.2. Plots of E_m vs pH were obtained with KaleidaGraph software, using slope values of either −59 mV/pH unit (corresponding to the uptake of 2 protons/disulfide reduced) or −29.5 mV/pH unit (corresponding to the uptake of 1 proton/disulfide reduced), as described by Chivers et al. (*19*). In all cases, the fit to the data obtained for two intersecting straight lines with these slopes was significantly better than the fit obtained for a single straight line with a slope of −59 mV/pH unit or the fit obtained for two straight lines, one with a slope of −59 mV/pH unit and one with a slope of 0 mV/pH unit (corresponding to a pH-independent process).

RESULTS

Figure 2 shows the results (○) of a representative oxidation−reduction titration for the recombinant wild-type sorghum MDH at pH 7.0. Four independent titrations at this pH gave an average value for E_m of −330 mV, with an average deviation of ±10 mV. All titrations of the wild-type enzyme gave an excellent fit to the Nernst equation for a single two-electron redox process, and attempts to fit the data with two $n = 2$ components gave no improvement in the quality of the fit. Titrations of the wild-type enzyme carried out at other pH values over the range from 6.0 to 10.0 also gave excellent fits to the Nernst equation for a single two-electron redox process. Figure 2 also shows a titration (●) of the C29S/C207A/C365A/C377A quadruple mutant that cannot form any regulatory disulfides and has previously been shown to be permanently active (*11*). In contrast to the behavior observed for the wild-type MDH, the activity of this mutant is, as expected, high and essentially constant at all E_h values tested and this behavior provides an important control for the titrations of E_h-dependent processes. Activity that was high and independent of E_h was also observed for the quadruple mutant at pH 6.0, 8.0, and 9.0.

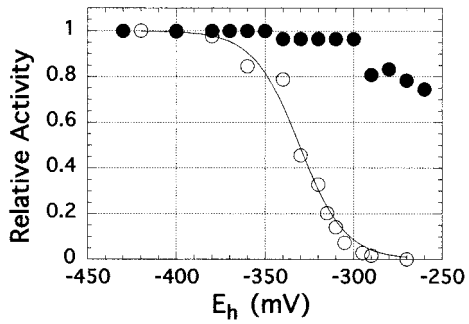

FIGURE 2: Oxidation−reduction titration of wild-type MDH and its C29S/C207A/C365A/C377A permanently active mutant at pH 7.0. Redox titrations and MDH enzyme assays were carried out as described under Materials and Methods. The redox equilibrium time was 10 min for the wild-type enzyme (○) and 3 min for the quadruple mutant (●). The line drawn through the points obtained with wild-type MDH represents the best fit to the Nernst equation with values of 2.0 for n and −330 mV for E_m.

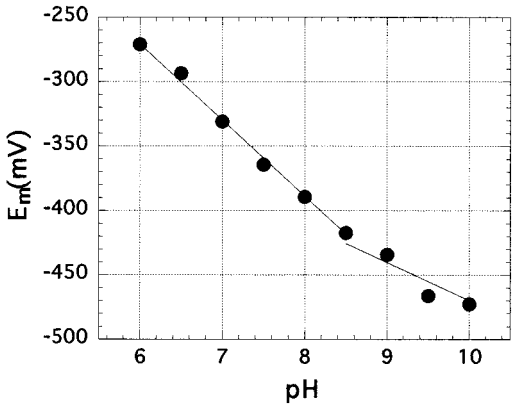

FIGURE 3: Effect of pH on E_m for wild-type MDH. Reaction conditions were as described in Figure 2.

Figure 3 shows the pH dependence of E_m for wild-type MDH over the range from pH 6.0−10.0 (the enzyme was not sufficiently stable at pH values > 10 to allow extending the titration to more alkaline values). Although the data obtained over the range from pH 6.0 to 8.5 give a good fit to the −59 mV/pH unit dependence expected for a process in which 2 protons are taken up per disulfide reduced (*19*), the data obtained at higher pH values give a better fit to the −29.5 mV/pH unit dependence expected for a process in which only one proton is taken up per disulfide reduced (*19*). The best fit for the total data set shown in Figure 3 is obtained with two straight lines: one, with a −59 mV/pH unit slope, for the region below pH 8.5 and a second, with a slope of −29.5 mV/pH unit, for the region from pH 8.5 to 10.0. The intersection of these two lines, which occurs at pH 8.7 ±

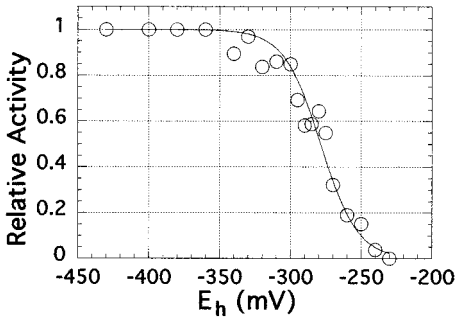

FIGURE 4: Oxidation−reduction titration of the C207A/C365A/C377A mutant of MDH at pH 7.0. Redox titrations and MDH enzyme assays were carried out as described under Materials and Methods, with a redox equilibration time of 3 min. The line drawn through the points represents the best fit to the Nernst equation with values of 2.0 for n and −280 mV for E_m.

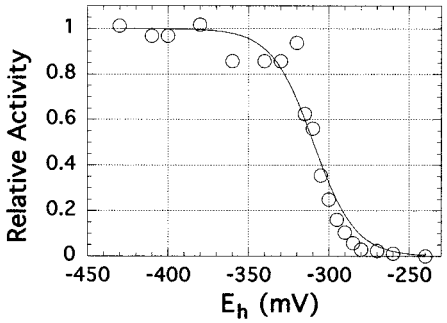

FIGURE 5: Oxidation−reduction titration of the activity of the ΔN mutant of MDH at pH 7.0. Redox titrations and MDH enzyme assays were carried out as described under Materials and Methods, with a redox equilibration time of 3 min. The line drawn through the points represents the best fit to the Nernst equation with values of 2.0 for n and −310 mV for E_m.

0.2, is likely to represent the pK_a for the more acidic cysteine in the reduced form of the regulatory disulfide being titrated (*19*). However, as the fit in the pH region above the putative pK_a relies on only four data points, this conclusion must be somewhat tentative.

If, as suggested in the introduction, more than one disulfide is involved in the activation of NADP-MDH, the fact that only a single redox component is detected in titrations of the wild-type enzyme could be due to the fact that the enzyme becomes active only when the most negative disulfide is reduced or, alternatively, to the fact that all of the disulfides involved in the activation process have E_m values too similar to be resolved in our measurements (which require a difference in E_m value greater than 20 mV for unambiguous resolution of two $n = 2$ components during a single titration). In an attempt to distinguish between these alternatives, redox titrations were carried out on a series of MDH mutants, each of which should be capable of forming only a single regulatory disulfide. Figure 4 shows the results of a titration, carried out at pH 7.0, of the C207A/C365A/C377A mutant of MDH, a form of the enzyme that contains the N-terminal C24/C29 bridge as the only disulfide. The data give a good fit to the Nernst equation for a single $n = 2$ redox component with $E_m = -280 \pm 10$ mV, a value significantly more positive than the $E_m = -330$ mV value obtained for the wild-type enzyme (i.e., the 50 mV difference between these values is well above the experimental uncertainty involved in the measurements). Titrations over the pH range from 6.0 to 10.0 indicated that the E_m value obtained for activation of this MDH mutant was 50 mV more positive than that obtained for the wild-type enzyme at all pH values in this range. The E_m vs pH dependence observed for this mutant (not shown) was similar to that determined for wild-type MDH, with an apparent pK_a of pH 8.5 ± 0.2 for the more acidic cysteine involved in C24/C29 disulfide formation.

Figure 5 shows the results of a titration of the ΔN mutant of MDH (this form of the enzyme has its 33 amino acid long N-terminal portion containing C24 and C29 deleted and has the C365/C377 bridge as the only regulatory disulfide remaining), carried out at pH 7.0. The E_m value of −310 ± 10 mV measured for the ΔN mutant is 30 mV more negative than that observed for the C207A/C365A/C377A mutant and agrees, within the experimental uncertainty, with the value measured for the wild-type enzyme (i.e., the 20 mV difference between the two values is equal to the maximum expected uncertainty for the difference between two values each with a ±10 mV uncertainty). The E_m value for the ΔN mutant is 30 mV more negative than that measured for the C207/C365A/C377A mutant at all pH values between 6.0 and 10.0. These results are consistent with the hypothesis of Hatch and Agostino (*4*) that there exists a preregulatory disulfide that has a more positive E_m value than does the regulatory disulfide and expands this concept by identifying the C24/C29 disulfide as the one most likely to be the more positive preregulatory group and the C-terminal C365/C377 disulfide as the more negative component. The E_m vs pH behavior determined for the ΔN mutant of MDH was quite similar to that observed for the wild-type enzyme, with an intersection of the −59 mV/pH unit and −29.5 mV/pH unit straight-line portions of the plot at pH 8.5 ± 0.2. Results similar to those observed for the ΔN mutant of MDH were also observed for the C24S/C29S mutant of MDH (a C24S/C29S double mutant that, like the ΔN mutant, has only the C-terminal C365/C377 disulfide), with $E_m = -300 \pm 10$ mV at pH 7.0 and an apparent pK_a of 8.4 ± 0.2 for the more acidic cysteine.

In the framework of the current working hypothesis that the C24/C29 disulfide initially present in the enzyme is replaced by a C24/C207 disulfide during the activation process (*3*, *7*), it was of interest to titrate the C29S/C365A/C377A triple mutant with only the C24/C207 disulfide remaining. Figure 6 indicates that the data give a good fit to the Nernst equation for a single $n = 2$ component with $E_m = -310 \pm 10$ mV at pH 7.0. Thus, the C24/C207 disulfide, like the C365/C377 disulfide, appears to have a more negative E_m value than the C24/C29 N-terminal disulfide. The E_m vs pH behavior of the C29S/C365A/C377A mutant was qualitatively similar to that observed for wild-type MDH, but with an apparent pK_a for the more acidic cysteine of 8.1 ± 0.2. The redox behavior of the C207A single mutant of MDH (a version of the enzyme that, like the wild-type enzyme, contains both the N-terminal and C-terminal disulfides but that, unlike the wild type, cannot form a C24/C207 disulfide), is essentially identical to that of the wild-type enzyme in terms of both its E_m vs pH behavior and its E_m value at pH 7.0 of −320 ± 10 mV (data not shown).

It has been reported that the presence of $NADP^+$, which is known to inhibit the activation of the enzyme (*11*, *20*), affects the equilibrium constant for the reaction between

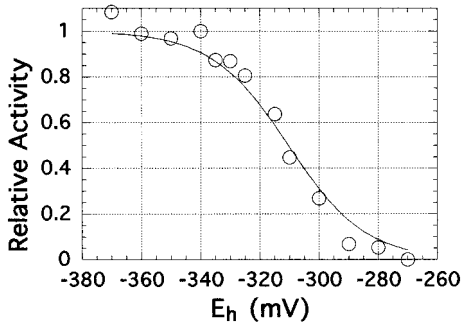

FIGURE 6: Oxidation−reduction titration of the C29S/C365A/C377A mutant of MDH at pH 7.0. Redox titrations and MDH enzyme assays were carried out as described under Materials and Methods, with a redox equilibration time of 3 min. The line drawn through the points obtained represents the best fit to the Nernst equation with values of 2.0 for n and −310 mV for E_m.

spinach NADP-MDH and DTT in a manner consistent with a 12 mV shift to a more negative value in the E_m for activation of the enzyme (*9*; R. Scheibe, personal communication). Under conditions where the inhibitory effect of $NADP^+$ on activation was clearly observed, we found no effect of the presence of $NADP^+$ on the E_m for activation of the wild-type enzyme. However, the experimental uncertainty inherent in our E_m determinations would not have allowed us to detect an E_m shift of only 12 mV, even if sorghum MDH behaved in a manner identical to that reported (*9*) for the spinach enzyme. Thus, although we did not observe any effect of $NADP^+$ on the redox properties of wild-type sorghum MDH, our results are not inconsistent with the earlier report of Faske et al. (*9*) that a small, $NADP^+$-dependent shift in the E_m for activation effect can be observed with the spinach enzyme.

A recent report by Ruelland et al. (*14*) describes the properties of a set of mutants of sorghum MDH that were used to provide evidence that the negative charges on the two amino acid residues at the C-terminus of the enzyme (i.e., the C-terminal carboxyl group of valine and the side-chain carboxyl group of the penultimate glutamate) allow the docking of these two amino acids into the active site, where they serve as a regulatory internal inhibitor (*14*). These mutations, combined with the elimination of the N-terminal disulfide(s), yield proteins exhibiting a high activity in the oxidized form, indicating that the local environment of their C-terminal disulfide has been substantially modified from that present in the wild-type enzyme. Thus it was of interest to examine the redox activation of a representative mutant used in the work of Ruelland et al. Titrations of the C29S/C207A/E387Q mutant of MDH (a form of the enzyme that has C365/C377 as the only disulfide and in which the negative charge on the penultimate amino acid has been removed by the site-specific conversion of glutamate to glutamine) gave E_m values of -275 ± 10 mV at pH 7.0 and -340 ± 10 mV at pH 7.9. These E_m values for the C-terminal C365/C377 disulfide in the C29S/C207A/E387Q mutant of MDH are 25−55 mV more positive than the values obtained for this disulfide in titrations of the wild-type enzyme and of its C24S/C29S, ΔN, and C207A mutants. It thus appears that the conformational change that likely results from the elimination of the negative charge of the side chain at position 387 in sorghum MDH (*14*) has an experimentally significant effect on the redox properties of the C-terminal regulatory disulfide.

Table 1: Summary of the Oxidation−Reduction Midpoint Potentials of Wild-Type NADP-MDH and NADP-MDH in Which Cysteines Involved in Regulation Have Been Altered by Site-Directed Mutagenesis[a]

enzyme preparation	disulfide titrated	E_m at pH 7.0 (mV)
wild type	C365/C377	−330
C207A	C365/C377	−320
ΔN (N-terminal deletion)	C365/C377	−310
C24S/C29S	C365/C377	−300
C207A/C365A/C377A	C24/C29	−280
C29S/C365A/C377A	C24/C207	−310

[a] In the case of the wild type and the C207A mutant, where two disulfides are present, the value shown is for the more negative.

DISCUSSION

The data presented above provide support for the idea, orginally proposed by Hatch and Agostino (*4*), that the pair of regulatory disulfides present initially in the oxidized, inactive form of NADP-dependent chloroplast MDH have different oxidation−reduction midpoint potentials. This study has now also provided the first evidence that the regulatory disulfide located in the N-terminal region of the enzyme (C24/C29 in the sorghum protein) has a more positive E_m value than does the regulatory disulfide found in the C-terminal portion of the enzyme (C365/C377 in the sorghum protein). It should also be pointed out that the titration data presented above for a C29S/C365A/C377A MDH mutant, where C24/C207 is the only possible regulatory disulfide present, provide the first thermodynamic evidence for the existence of this disulfide in MDH and demonstrate that the enzyme is not fully active unless the C24/C207 disulfide is fully reduced.

Our value for the E_m of the C24/C29 disulfide, −280 mV at pH 7.0, comes exclusively from titrations of the C207A/C365A/C377A mutant of MDH, as this is the only mutant of the enzyme available to us that contains C24/C29 as the only disulfide. Titrations of two MDH mutants, ΔN (the mutant with the N-terminal 33 amino acids deleted) and C24S/C29S, that contain the C-terminal C365/C377 bridge as the only disulfide gave an average E_m value for activation of −305 mV, more negative than the −280 mV value obtained for the C207A/C365A/C377A mutant. As a considerable body of independent evidence indicates that both the C24/C29 and C365/C377 disulfides must be reduced before MDH becomes detectably activated (*1*), one would expect that the E_m value determined for activation of the wild-type enzyme would be the same as the E_m value for the more negative of these disulfides. Thus, we have assigned the E_m value of −330 mV obtained for activation of the wild-type enzyme to the C365/C377 disulfide. A similar E_m value, −320 mV, was obtained in titrations of the C207A mutant and, following the same reasoning used for assigning the E_m value observed for the wild-type enzyme to the more negative of the C24/C29 and C365/C377 disulfides, we attribute this value to the C365/C377 disulfide.

Table 1 summarizes the results we have obtained in redox titrations of the wild-type enzyme and of mutants in which the cysteines involved in regulation of the enzyme have been replaced by site-directed mutagenesis. The average E_m value for the C365/C377 disulfide from these four sets of titrations

is −315 mV, more negative than the value we have measured for the C24/C29 regulatory disulfide by an amount greater than the experimental uncertainties in the measurements. The differences in E_m value for the C365/C377 disulfide in the four different forms of the enzyme utilized in this study are probably within the experimental uncertainties of our methodology. However, if instead they represent real differences they could result from subtle differences in the environment of this disulfide in the mutated enzymes compared to wild-type MDH (an average value of −305 mV for the proteins having only the C-terminal disulfide vs −325 mV for proteins having both N-terminal and C-terminal disulfides). In fact, it has been suggested (*6*) that the constrained structure of the fully oxidized form of the enzyme may make both the N-terminal and C-terminal regulatory disulfides more difficult to reduce than would be the case for a the more relaxed structure likely to be present if one of the disulfides were absent. This hypothesis (*6*), which is consistent with the possibility of cooperativity between disulfide reduction in wild-type MDH (*21*), could explain a positive shift in the E_m of the C365/C377 disulfide such as that observed (see above) with the C29S/C207A/E387Q mutant. This highly deregulated mutant, which exhibits a high spontaneous activity in the oxidized form, has the E_m of the C-terminal disulfide shifted to −275 mV. The change in structure caused by this mutation, in which the penultimate C-terminal negative charge has been eliminated, is likely to be greater than that present in C/S or C/A mutations, which simply mimic the natural reduction of the disulfides.

Recent measurements in our laboratories have established that the E_m values for the two chloroplast thioredoxins, thioredoxin *f* (spinach and pea) and thioredoxin *m* (spinach and *Chlamydomonas reinhardtii*), are −290 and −300 mV, respectively, at pH 7.0 (*16*). An identical value of −300 mV has been reported for maize thioredoxin *m* at pH 7.0 (*8*) and a somewhat more positive value (i.e., 20 mV more positive when comparing E_m values obtained at pH 7.9) has been reported for spinach thioredoxin *f* (*22*; D. Ort, personal communication). It is not possible to unambiguously calculate thermodynamic driving forces for the various steps in the activation of sorghum MDH by thioredoxin because it is not yet clear which of the two chloroplast thioredoxins is responsible for the activation of MDH in vivo (*23, 24*) and because E_m values for the sorghum chloroplast thioredoxins have not been measured. However, if the sorghum thioredoxins have E_m values at pH 7.0 in the −290 to −300 mV range and the −280 mV E_m value we have measured for the C24/C29 disulfide in the C207A/C365A/C377A mutant of MDH accurately reflects the value for this disulfide in the wild-type enzyme, then reduction of the N-terminal disulfide during activation of MDH will be a thermodynamically favorable process, although with only a small driving force of some 10−20 mV. Reduction of the C365/C377 disulfide, if it has a E_m value of −330 mV, by a chloroplast thioredoxin will be thermodynamically unfavorable by some 30−40 mV. At redox equilibrium, a high ratio of reduced to oxidized thioredoxin would be required to overcome this 30−40 mV gap, consistent with earlier findings that the extent of thioredoxin reduction is greater than the extent of MDH reduction during the activation process (*21, 26*). Reduction of the $E_m = -310$ mV C24/C207 disulfide by either thioredoxin *f* or *m* would also be slightly endergonic.

In attempting to calculate the thermodynamic driving force for reduction of any of the regulatory disulfides in MDH by chloroplast-located thioredoxins, it should be kept in mind that the E_m values for the reactants are pH-dependent (see above; *16*) and that the pH of the chloroplast stromal space where these proteins are located (*27*) rises during illumination by almost 1 pH unit, from a pH close to 7.0 to one near 7.9 (*25*). Both thioredoxins *f* and *m* have been shown to display the same −59 mV/pH unit dependence of E_m from pH 7.0 to 8.0 (*16*) that is observed for wild-type MDH (see Figure 2) and its mutants over most of the pH range from 7.0 to 8.0. The E_m vs pH plots measured for wild-type MDH and all of the mutants examined do show a change in slope in E_m vs pH plots from −59 to −29.5 mV/pH unit at pH values between 8.0 and 8.7, and thus there may be a small effect on the thermodynamic driving forces for the reduction of MDH regulatory disulfides by either thioredoxin *f* or *m* at the alkaline extreme of the light-induced changes in stromal pH.

Limitations on obtaining data at very alkaline pH values, arising from the instability of the enzyme in this pH region, make the assignment of possible pK_a values for the wild-type enzyme and its site-specific mutants somewhat uncertain. However, if one assumes that the best-fit intersections of the two straight-line segments of E_m vs pH profiles for MDH represent the pK_as for the more acidic cysteine in the reduced form of each disulfide (*19*), then each of the three regulatory cysteine pairs in MDH has one cysteine with a pK_a in the range between 8.1 and 8.7. It also seems likely, for each of the three cysteine pairs involved in the regulation of MDH, that one of the cysteines must have a significantly more alkaline pK_a than does the other cysteine in the pair. This conclusion is based on the fact that if the pK_a values for both cysteines in each regulatory pair were equal (or experimentally indistinguishable), then no protons would be taken up on disulfide reduction at pH values above this pK_a and one would have observed a transition from a −59 mV/pH unit slope to a pH-independent region (*19*) instead of to the −29.5 mV/pH unit dependence actually observed. The 0.4 pH unit difference in pK_a values for the more acidic cysteines of the C24/C29 disulfide (p$K_a = 8.5 \pm 0.2$) and the C204/C207 disulfide (p$K_a = 8.1 \pm 0.2$) is just at the limit of the combined uncertainties in the two measurements. If this difference is in fact statistically significant, the fact that C24 is a constituent of both the C24/C29 and C24/C207 regulatory disulfides and the fact that the more acidic cysteine in a regulatory pair is likely to be the one involved in the formation of an intermolecular disulfide between thioredoxin and a thioredoxin-regulated enzyme (*7, 28, 29*), allow a preliminary assignment of these pK_a values to specific cysteines on the basis of recent evidence from structural and mutagenesis studies. Thus, as it appears likely that C207, from the C24/C207 pair, provides the reactive thiol (*7*), we propose that the pK_a of the thiol group of C207 is 8.1. Evidence from the X-ray crystal structure of NADP-MDH suggests that C29, rather than C24, is the initial site of reaction between thioredoxin and the C24/C29 pair (*5, 6*), supporting an assignment of the pK_a value of 8.5 to the thiol group of C29. Confirmation of these pK_a values by structural information and the results of chemical modification and additional site-directed mutagenesis experiments can have

important implications in understanding the complex activation of MDH.

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