Engineering a Domain-Locking Disulfide into a Bacterial Malate Dehydrogenase Produces a Redox-Sensitive Enzyme

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ABSTRACT Light-dependent reduction of cystine disulfide bonds results in activation of several of the enzymes of photosynthetic carbon metabolism within the chloroplast. We have modeled the tertiary structure of four of these light-activated enzymes, namely NADP-linked malate dehydrogenase, glyceraldehyde-3-P dehydrogenase, fructosebisphosphatase, and sedoheptulosebisphosphatase, and identified cysteines in each enzyme that might be expected to form inactivating disulfide bonds (Li, D., F. J. Stevens, M. Schiffer, and L. E. Anderson, 1994. *Biophys. J.* 67:29–35). We have now converted two residues in the *Escherichia coli* NAD-linked malate dehydrogenase to cysteines and produced a redox-sensitive enzyme. Oxidation of domain-locking cysteine residues in the mutant enzyme clearly mimics dark inactivation of the redox-sensitive chloroplast dehydrogenase. This result is completely consistent with our proposed mechanism.

INTRODUCTION

The NADP-linked malate dehydrogenase (EC 1.1.1.82) in chloroplasts is reductively activated by a process that involves transfer of electrons from the photochemical apparatus via ferredoxin, ferredoxin-thioredoxin reductase and thioredoxin to a disulfide bond on the target enzyme (Anderson, 1986; Buchanan, 1991; Buchanan et al., 1994; Scheibe, 1991). We have identified a pair of thioredoxin-accessible cysteines positioned on opposing domains in the chloroplast enzyme by homology modeling, and we have postulated that the cystine disulfide bond that would be formed by oxidation of these cysteine residues would restrict motion around the hinge connecting the two domains with a resultant decrease in activity (Li et al., 1994). The other three light-activated enzymes that we are currently able to model are also two-domain enzymes, and in each case the potential disulfide might be expected to directly or indirectly affect the interdomain movement necessary for catalysis. We reasoned that if light activation involves these cysteine residues and this mechanism, we should be able to construct redox-modulatable enzymes from the enzymes of non-photosynthetic species. We chose the NAD-linked malate dehydrogenase (EC 1.1.1.37) from *Escherichia coli*, which is insensitive to oxidizing and reducing agents and does not contain any of the Cys residues present in the five chloroplast malate dehydrogenases sequenced to date, as our first enzyme for mutation.

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Abbreviations and trivial names used: diamide, diazinedicarboxylic acid bis(*N,N*-dimethylamide); IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate.

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MATERIALS AND METHODS

Expression of *E. coli* malate dehydrogenase and introduction of Cys codons

The native *E. coli* malate dehydrogenase gene was cloned into the *XbaI*/*HindIII* sites of the expression vector pASK40 (Skerra et al., 1991) by polymerase chain reaction (PCR) (Clackson et al., 1991). Primers were designed to generate a protein identical to the native enzyme for which the crystal structure has been reported (Hall et al., 1992). The sense primer TATCATTCTAGATACGAGGGCAAAAATGAAAGTCGCAGTCC incorporated the *XbaI* site (which includes a stop codon that terminates translation of the vector's β-galactosidase) followed by a ribosome binding site and 16 bases of the N-terminal DNA sequence of *mdh* (McAlister-Henn et al., 1987). The antisense primer incorporated the *HindIII* site and the C-terminal sequence of the gene. Vent DNA polymerase (New England Biolabs, Beverly, MA) was used to insure fidelity. Cys codons were substituted into the DNA sequence by the method of Kunkel et al. (1987). *E. coli* strain CJ236 (Kunkel et al., 1987) was used to generate deoxyuracil-substituted DNA, and single-stranded DNA was isolated using the helper phage M13K07 (Veira and Messing, 1987). For mutant L305C, the oligonucleotide ATTAACGAACTCTTGGCCACAGGCGATATCTTTCTT was used to replace the codon for Leu305 (CTG) in the wildtype construct by a codon for a Cys (TGT) and to add an *MscI* restriction site (TGGCCA). For the double mutant N122C-L305C, the oligonucleotide AATTGCAACTGTTGTACAAACCGGGTTAGTGAT was used to replace the codon for Asn 122 (AAC) in the L305C construct by a codon for a Cys (TGT) and to add a *BsrgI* restriction site (TGTACA). The mutations were confirmed by sequencing. *mdh*⁻ *E. coli* strain BHBIII (kindly provided by Deborah Breiter and Leonard Banaszak, University of Minnesota) was transformed (Sambrook et al., 1989) with the pASK40 plasmid carrying the appropriate construct.

Selection

Transformants were selected on Luria-Bertani medium (Sambrook et al., 1989) containing 100 μg ml^{-1} ampicillin and 10 μg ml^{-1} kanamycin. Single colonies were added to 1.5 ml of Luria-Bertani liquid medium containing the antibiotics and cultured overnight at 37°C. 15 μl of these liquid cultures were used to inoculate 1.5 ml of the culture medium and grown at 37°C to log phase (~3 h), after which IPTG was added to 1 mM to induce expression of malate dehydrogenase. 10 h later the cells were collected by centrifugation (10,000 × *g*, 1 min), suspended in 0.5 ml of 50 mM Tris-HCl, pH 7.9, 0.05% NaN_3, 1 mM EDTA-K, 0.2% PMSF, 0.1 mg ml^{-1} chicken lysozyme (lysis buffer) and placed on ice for 2 h. Lysis was completed by

freezing (−20°C). After at least 30 min the lysate was thawed and 150 μl 50 mg ml^{-1} streptomycin was added. After 10 min (at 0°C) the mixtures were centrifuged (10,000 × *g*, 5 min), the supernates were treated with DTT (under N_2) or diamide for 30 min at 0°C, and the activity assayed.

Purification of malate dehydrogenase

A single colony of the *E. coli* transformant was inoculated directly into 800 ml Luria-Bertani medium containing antibiotics. When the turbidity was equivalent to A_{600} 0.5, IPTG was added to 1 mM to induce expression. 10 h later the cells were collected by centrifugation (5,000 × *g*, 10 min); resuspended in 10 ml of the lysis buffer, which was 1 mM in 2-mercaptoethanol (10 mM for the N122C-L305C mutant) and contained 1 mg ml^{-1} lysozyme; allowed to stand on ice for 1 h, and then frozen (−20°C). After at least 30 min the lysate was thawed and 300 mg streptomycin was added. After 15 min at 0°C the lysate was centrifuged (27,000 × *g*, 10 min) and the supernate was made to 35% saturation by addition of saturated $(NH_4)_2SO_4$. The solution was allowed to stand on ice for 1 h and centrifuged (15,000 × *g*, 10 min). The supernate was made to 65% saturation by addition of saturated $(NH_4)_2SO_4$, allowed to stand on ice for 1 h and centrifuged, as above. The pellet was resuspended in 5 ml of 10 mM Hepes-K, pH 7.4, 1 mM EDTA-K, 1 mM 2-mercaptoethanol (10 mM for the N122C-L305C mutant), dialyzed overnight with one change against 1 L of the same buffer, and applied to a 10-ml Procion Blue HERD Sephacryl (Skrukrud et al., 1991) column. The column was washed with 30 ml of the Hepes buffer, and developed with a linear 0–0.5 M NaCl gradient (250 ml in the Hepes buffer made 0.02% in PMSF). The fractions with the highest activity were pooled. There were faint traces of other proteins (<5% of the Coomassie-staining material) evident after SDS gel electrophoresis. Yields were ~1.5 mg enzyme/L culture for overexpressed wildtype, 6 mg/L for L305C, and 2 mg/L for N122C-L305C.

Estimation of kinetic parameters

For estimation of K_m and maximal velocity values cuvettes contained 100 mM potassium phosphate, pH 7.5, and, when oxalacetate was varied, 0.3 mM NADH. For the N122C-L305C mutant malate dehydrogenase oxalacetate concentrations (12 total) were varied between 0.05 and 3 mM. Oxalacetate concentrations were 10-fold lower for the other three enzymes. When NADH was varied and the enzyme was the N122C-L305C mutant the oxalacetate concentration was 3 mM. For the other three enzymes the oxalacetate concentration was 0.33 mM. NADH was varied (12 concentrations)

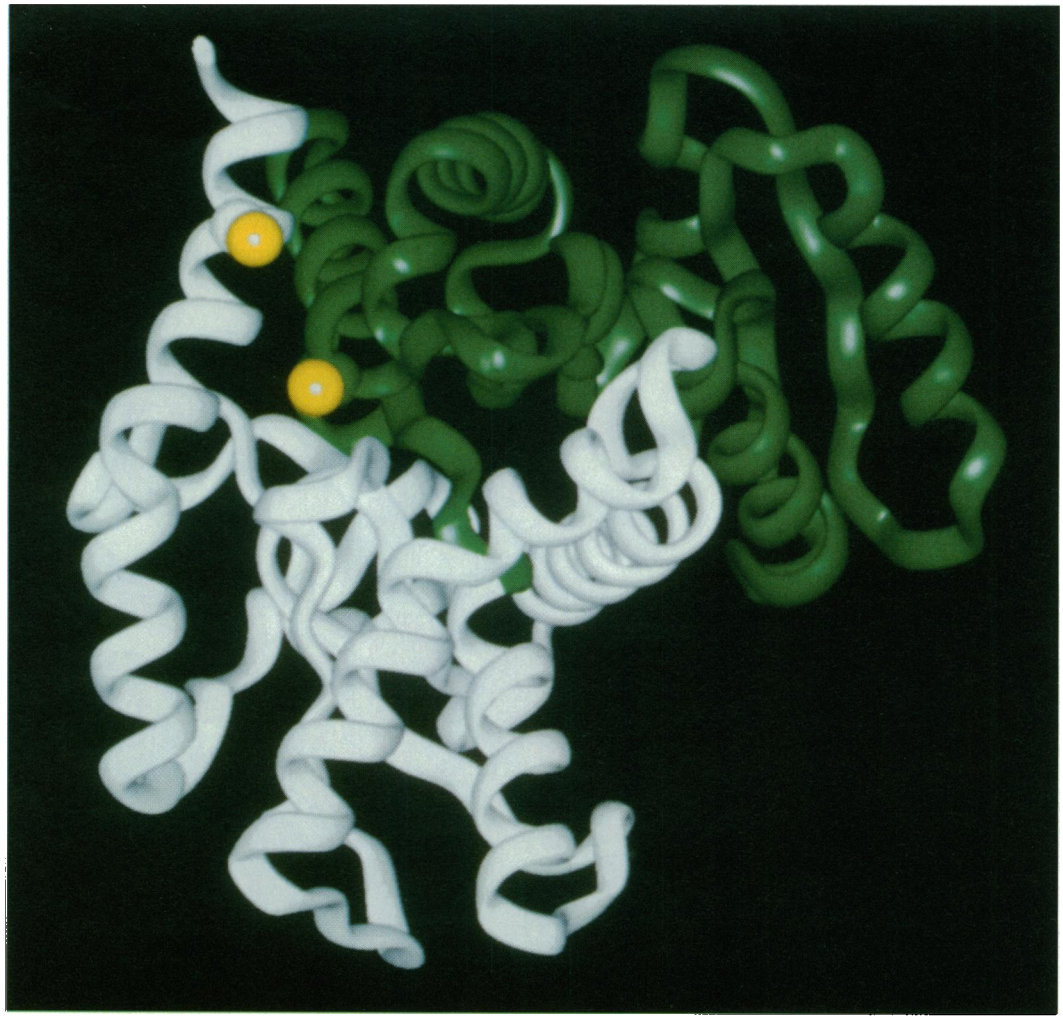

FIGURE 1 Tertiary structure of *E. coli* malate dehydrogenase with native residues replaced by cysteines introduced by site-directed mutagenesis. The carbon substrate-binding domain is shown in white and the nucleotide-binding domain is shown in green. The cysteines shown in creatine phosphokinase representation in yellow are 305 in the long helix on the left and 122 in the short helix on the right. Mutants containing these two cysteine residues were redox-sensitive. The distance between the Cys-305 and 122 α-carbons is 7.6 Å. We used coordinates (Entry 2CMD, version of 31 October 1993) for *E. coli* malate dehydrogenase (Hall et al., 1992) obtained from the Protein Data Bank at Brookhaven National Laboratory.

TABLE 1 Effect of DTT and diamide on malate dehydrogenase activity in crude extracts

Treatment	Malate dehydrogenase activity (units mg protein^{-1})		
	Wildtype	L305C	N122C-L305C
None (control)	49 ± 6	49 ± 10	5 ± 1
10 mM DTT	44 ± 6	54 ± 6	18 ± 3
1 mM Diamide	58 ± 5	50 ± 11	2.0 ± 0.6
DTT reversal of diamide inhibition	52 ± 5	45 ± 10	7 ± 1

The concentration of DTT or diamide in the assay cuvette was 1/100 of the concentration during treatment. DTT was added directly to the cuvette (5 mM in cuvette) to reverse the effect of diamide. Results are means from duplicate activity determinations for six single colony transformants for each mutant and for overexpressed wildtype.

between 0.005 and 0.3 mM. The data were analyzed with the computer program of Hanson et al. (1967). Weighted mean values and SEM were estimated with 1/variance as weighting factor.

Activity assay

Malate dehydrogenase activity was routinely assayed in the direction of NADH oxidation according to Murphey and Kitto (1969) on a Cary 210 or 219 (Varian, Sunnyvale, CA) recording spectrophotometer at 25°C.

Protein estimation

Protein was estimated by the method of Bradford (1976) with bovine serum albumin as standard.

Tertiary structure diagrams

Tertiary structure diagrams were displayed and made with INSIGHT II (Biosym Technologies, San Diego, CA) on a Silicon Graphics workstation.

Energy minimization

Minimization was performed on malate dehydrogenase dimers generated by symmetry operation on the Protein Data Base 2CMD monomer. Residues at positions 305 and 122 in both subunits were computationally converted to Cys with INSIGHT II. The separation of S_γ atoms was reduced by manual rotation around the C_α—C_β bond. In one of the monomers, a bond was forced between the sulfurs. Using the Discover Molecular Dynamics Package (Biosym Technologies) (cutoff of 16 Å for calculating electrostatic interactions, dielectric 60) all heavy atoms were initially tethered at 2000 kcal Å^{-1}. The tethering force was gradually reduced over 1500 steps of steepest descent minimization. A complex conjugates method was used for subsequent minimization until convergence criterion was achieved. Completion criterion was a maximum gradient <0.001 kcal Å^{-1}.

RESULTS AND DISCUSSION

Our model predicts that in the chloroplast malate dehydrogenase the Cys residue corresponding to *E. coli* residue 305 forms a domain-locking disulfide with the Cys residue at the N- or C-terminus of helix 5. To test this hypothesis we constructed a double mutant in which the residues corresponding to 305 in helix 9 and 122 in helix 5 were changed to Cys (Fig. 1). The selection of these residues for mutation was based on alignment of the tertiary structures of the pig and *E. coli* enzymes and on primary structure alignment of the chloroplast and *E. coli* enzymes. In the chloroplast enzymes, as

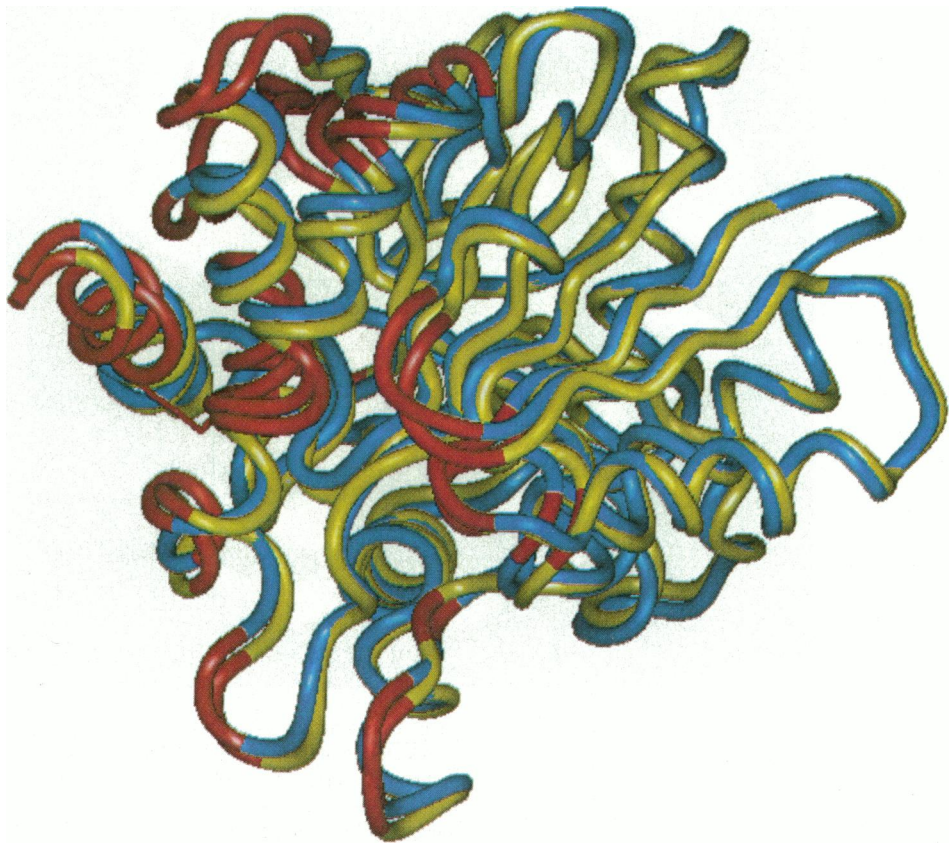

FIGURE 2 Overlay of reduced (blue) and oxidized (yellow) backbone structures. The average displacement was 0.95 Å with rms displacement 1.19 Å. Red indicates locations of α-carbons displaced by more than 1.4 Å as a result of energy minimization.

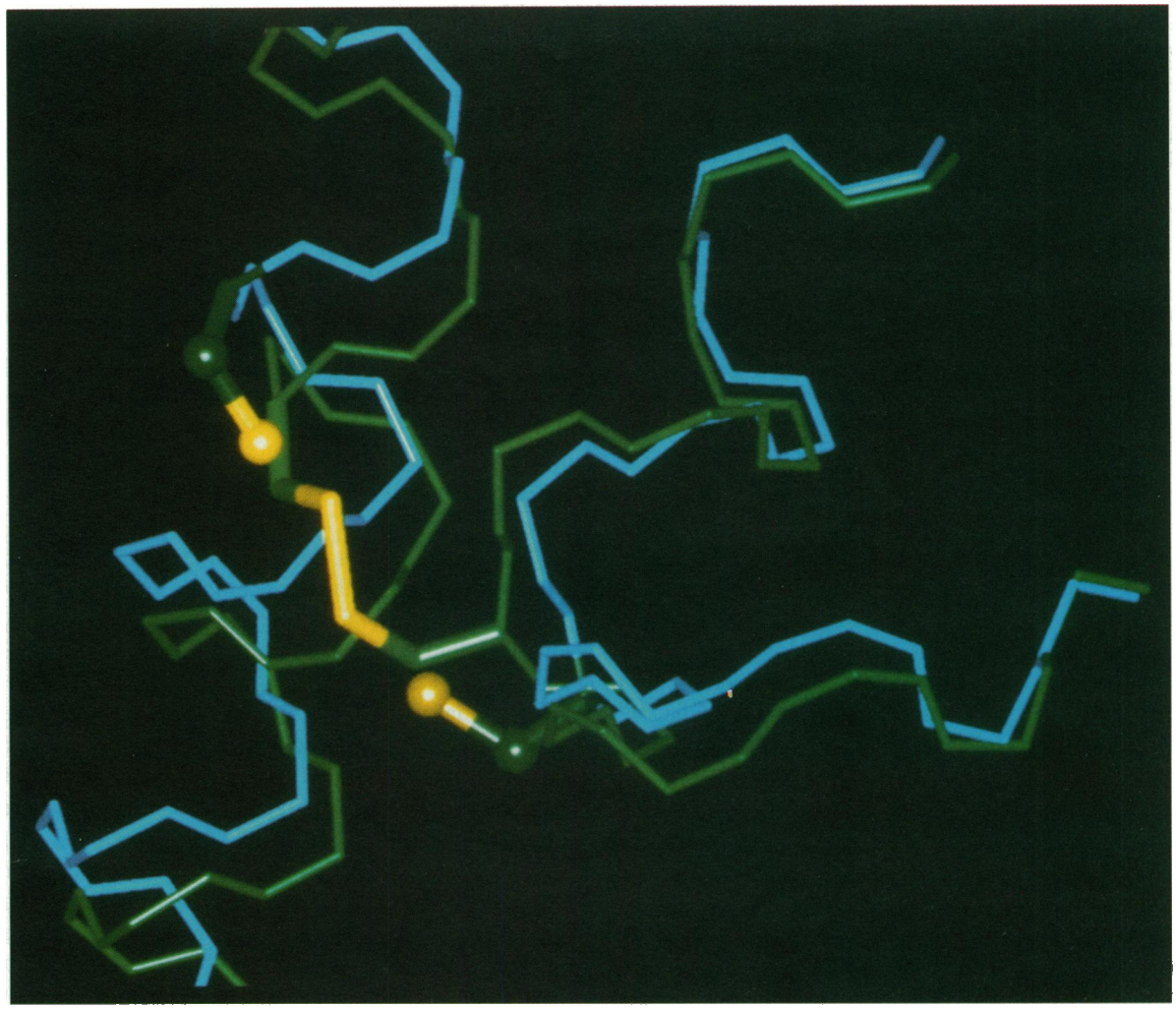

FIGURE 3 Orientation of Cys-122 and Cys-305 before (blue) and after (green) formation of disulfide bond. View is along helix 9 (*left*) and down helix 5 (*right*). Cys-122 and -305 in yellow. After formation of disulfide bond 7000 steps of steepest descent energy minimization and complex conjugates minimization to convergence, the disulfide bond length decreased to 2.0 Å. No water molecules were included in the energy minimization, which was intended only to test the feasibility of forming this disulfide bond.

modeled, rotation of helices 5 and 9 relative to one another is required for formation of a disulfide bond between the cysteines corresponding to residues 305 and 121 (Li et al., 1994). We mutated residue 122 rather than 121 to eliminate the necessity for this movement. The mutant enzyme is then, if our hypothesis is correct, a simplified version of the redox-sensitive chloroplast enzyme.

None of the introduced residues is within disulfide bonding distance of the three Cys residues in the native enzyme. Cys-122 C_α is 23.4 Å from Cys-109 C_α, 17.8 Å from Cys-113 C_α, and 14.2 Å from Cys-251 C_α. The C_β distances are 24.7, 19.5, and 14.2 Å, respectively. These separations are far greater than the expected C_α distance (6.5 Å) and C_β distance (4.5 Å) for protein cystines. The Cys-305 separations are even larger. Formation of disulfide bonds between the native enzyme Cys residues and either of the introduced Cys residues would require extreme rearrangement of the structure (not shown).

Consistent with our prediction the mutant enzyme is reductively activated by DTT and inactivated by diamide (Table 1). There was no evidence for an intersubunit disulfide bond when the diamide-treated malate dehydrogenase was subjected to denaturing gel electrophoresis (in the absence of DTT), but there was a new band on the gel running ~2 kDa ahead of the position of the reduced enzyme. A decrease in the Stokes radius is expected if there is a crosslinking disulfide between Cys-122 and Cys-305. Apparently there is a disulfide bond formed as a result of oxidation, which connects two Cys residues fairly distant from one another in the primary structure. Final identification of the introduced residues as the disulfide-bond-forming cysteines will require either experimental determination of the tertiary structure of the mutant enzyme or peptide mapping.

The computer-generated Cys sulfurs in the predicted structure (Fig. 1) are separated by 11 Å, and the side chains are not oriented toward each other. By rotating the two side chains this distance can be reduced to 5.8 Å. This conformation might seem to rule out the possibility of a disulfide bond. However, when we computationally bonded the sulfurs and did 1500 steps of energy minimization, the sulfurs

TABLE 2 Kinetic parameters of recombinant wildtype and mutant malate dehydrogenases

Varied substrate, values	Wildtype	L305C	N122C-L305C (DTT)	N122C-L305C (diamide)
K_m (μM)				
oxalacetate	49 ± 3 (1)	44 ± 4 (1)	340 ± 20 (1)	580 ± 20 (1)
NADH	61 ± 2 (4)	87 ± 5 (2)	56 ± 2 (2)	52 ± 3 (2)
K_m (mM)				
L-malate	2.6 ± 0.2 (2)		5.3 ± 0.3 (2)	6.8 ± 0.6 (2)
NAD	0.26 ± 0.03 (2)		0.38 ± 0.02 (2)	0.50 ± 0.03 (2)
k_{cat} (ms^{-1}, malate formation)	0.9 (1)	1.1 (2)	1.1 (1)	0.13 (1)
k_{cat} (s^{-1}, oxalacetate formation)	21 (2)		8.6 (2)	1.5 (2)

Number of determinations is given in parenthesis. We made three different K_m(oxalacetate) and K_m(NADH) estimates for each enzyme, under slightly different conditions. The values obtained in all cases were similar to those reported here. The N122C-L305C mutant enzyme was incubated in 10 mM DTT under N_2 on ice (DTT-treated) or in 1 mM diamide (diamide-treated) for 30 min before assay. K_m and k_{cat} values for the diamide-treated mutant are quite variable as a result of differences in the extent of inhibition. Pyridine nucleotide concentrations were varied in the k_{cat} estimations. The estimated error in the k_{cat} values (not shown) was 3% or less. For comparison, the K_m values for the light modulatable maize NADP malate dehydrogenase are 56 μM (oxalacetate) and 24 μM (NADPH) (Kagawa and Bruno, 1988) and the k_{cat} in the direction of malate formation calculated from data in (Kagawa and Bruno, 1988) using a subunit molecular mass of 40.9 kDa (Metzler et al., 1989) is 0.5 ms^{-1}.

moved to a reasonable disulfide bond distance of 2.0 Å (Fig. 2). These minimization calculations suggest that there is no significant steric hindrance to formation of a disulfide bond between Cys-122 and Cys-305.

Most of the rearrangement came from side chain rotation, but there was significant displacement of residues in the long helix (helix 9) downward from the position shown in Fig. 1 (Fig. 3). Considerable movement in this region of the protein is required; without this movement the two sulfhydryls would not be able to reach an orientation that would allow the disulfide bond formation suggested by our experiments. Introduction of potentially oxidizable Cys residues has already been used in assessing the extent of movement within proteins (see Careaga and Falke, 1992). The positions of inactivating redox-sensitive Cys residues, whether inserted or natural, should be useful in describing interdomain movement related to catalysis.

In contrast to the introduction of regulatory disulfide bonds into lysozyme (Matsumura and Mathews, 1989) and aspartate aminotransferase (Pan et al., 1994), the suitability of the mutation sites as defined by Sowdhamini et al. (1989) on the basis of orientation and proximity is not evident in the malate dehydrogenase crystal structure. Our experiments therefore suggest that designed redox sensitivity could be used to control the activity of many technologically important enzymes not now thought to be candidates for such modification.

There is a marked decrease in k_{cat} when the redox-sensitive mutant malate dehydrogenase is oxidized (Table 2). The K_m of the mutant enzyme for oxalacetate is an order of magnitude higher than the K_m of the wildtype enzyme whether treated with oxidant or reductant, while the K_m(NADH) of the N122C-L305C mutant enzyme is almost the same as the K_m of the wild type enzyme. Interestingly, the K_m of the redox-sensitive maize enzyme for oxalacetate is similar to that of the wildtype *E. coli* enzyme rather than to the K_m of the redox-sensitive mutant. There may then have been changes in the plant enzyme to compensate for the effect of the introduction of the two Cys residues juxtaposed on helices 5 and 9.

The pH optimum is about 6 for the N122C-L305C mutant, considerably lower than the optimum for the wildtype enzyme (Fig. 4). This might be expected; when both Cys residues are ionized they will repel one another with resultant structural distortion. As the proton concentration increases, the fraction of molecules in which both of the Cys residues are negatively charged will decrease, and hence distortion will be decreased. The pH dependency curves for the maize (Kagawa and Bruno, 1988), spinach, and pea (Ocheretina et al., 1993) chloroplast malate dehydrogenases are very similar to the pH dependency curves for the wildtype *E. coli* enzyme. Alterations in structure must then have occurred to accommodate the negative charge associated with the Cys residues in the chloroplast enzyme. Notably, truncation of the N-terminus of the spinach and pea enzymes lowers the pH optimum (Ocheretina et al., 1993).

The γ-subunit of *Synechocystis* 6803 CF_1 was recently made redox-sensitive by introduction of a nine-residue

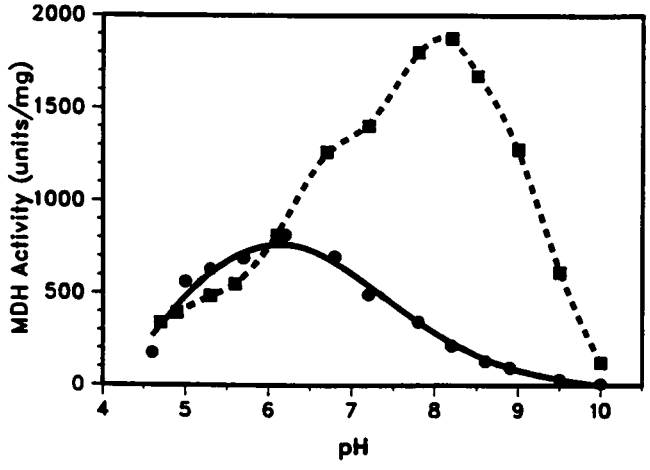

FIGURE 4 pH dependency of the recombinant wildtype (■) and DTT-treated N122C-L305C mutant (●) malate dehydrogenases. Cuvettes contained 100 mM potassium phosphate buffer, 0.3 mM oxalacetate, 0.15 mM NADH, and enzyme. (Note that this concentration of oxalacetate is only ~K_m for the mutant enzyme.) pH was measured after activity assay. DTT treatment as in Table 2.

stretch of amino acids, that contains two cysteines and is characteristic of the higher plant redox-sensitive CF_1 γ-subunit (Werner-Grüne et al., 1994). There are additional cysteines in the γ-subunit and it is therefore not clear whether both of the cystine-forming-Cys residues were added in the nine introduced amino acids. Clearly, it is possible to introduce biologically significant regulatory disulfides into redox-insensitive enzymes.

Mutants of the chloroplast malate dehydrogenase have been constructed in previous attempts to identify the cysteine residues involved in the redox-sensitive disulfides (Issakidis et al., 1993, 1994; Reng et al., 1993). Conflicting conclusions were reached by those investigators. (See discussion in Li et al. (1994).) No chloroplast malate dehydrogenase mutants involving the helix-5 Cys residues have been constructed. Our experiments are consistent with our model and implicate Cys-305 and one of the helix-5 cysteines as the likely redox-sensitive disulfide in chloroplast malate dehydrogenase. Clearly, locking the two domains by oxidation of Cys residues impairs catalysis, as predicted, in a reversible manner.

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