Mechanism of Light Modulation: Identification of Potential Redox-Sensitive Cysteines Distal to Catalytic Site in Light-Activated Chloroplast Enzymes

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ABSTRACT Light-dependent reduction of target disulfides on certain chloroplast enzymes results in a change in activity. We have modeled the tertiary structure of four of these enzymes, namely NADP-linked glyceraldehyde-3-P dehydrogenase, NADP-linked malate dehydrogenase, sedoheptulose bisphosphatase, and fructose bisphosphatase. Models are based on x-ray crystal structures from non-plant species. Each of these enzymes consists of two domains connected by a hinge. Modeling suggests that oxidation of two crucial cysteines to cystine would restrict motion around the hinge in the two dehydrogenases and influence the conformation of the active site. The cysteine residues in the two phosphatases are located in a region known to be sensitive to allosteric modifiers and to be involved in mediating structural changes in mammalian and microbial fructose bisphosphatases. Apparently, the same region is involved in covalent modification of phosphatase activity in the chloroplast.

INTRODUCTION

Several of the enzymes in the chloroplast stroma have the unique property of being activated by reduced thioredoxins in the light. They are regulated in such a way that the Calvin cycle will operate only in the light and not in the dark. These enzymes include NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, phosphoribulokinase, and NADP-dependent malate dehydrogenase. It is thought that activation is caused by conformational change resulting from breakage of a disulfide bond in the modulated enzyme by light-generated reduced thioredoxin (Buchanan, 1980; Anderson, 1986; Scheibe, 1991).

The multiple related but distinctive strategies through which disulfide bonds are utilized by chloroplast enzymes to link catalytic activity to the energy source appear to be a striking example of convergent evolution. Elucidation of the consequences of these disulfide bonds should provide new insight into the dynamics of the corresponding enzymes from non-plant species. Because there is no obvious sequence homology around the cys residues of the chloroplast enzymes (Scheibe, 1991), we decided to examine their positions in the predicted tertiary structure of the enzymes. We used the coordinates of homologous mammalian or bacterial forms of the enzymes as the basis for the models of the chloroplast enzymes. We were able to map the location of those cys residues for which there were corresponding residues in the reference structure. We did not attempt to predict the location of cys residues when they occurred in insertions or among the extra residues in N- and C-terminal extensions to the chloroplast enzymes.

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MATERIALS AND METHODS

Alignments were done with ALIGN Plus (2.0) (Scientific & Educational Software, State Line, PA) with parameters mismatch = 2; open gap = 4; and extend gap = 1. Coordinates were obtained from the Protein Data Bank at Brookhaven National Laboratory (Bernstein et al., 1977; Abola et al., 1987). The tertiary structure diagrams were displayed and made with INSIGHT II (Biosym Technologies, San Diego CA). No energy minimization procedures were used, and no manual adjustments were made.

RESULTS AND DISCUSSION

Glyceraldehyde-3-P dehydrogenase

We first modeled the tertiary structure of chloroplastic glyceraldehyde-3-P dehydrogenase. The chloroplast enzyme is a tetramer composed of either four identical subunits (A_4) or of two A and two slightly longer B subunits (A_2B_2). Like other glyceraldehyde-3-P dehydrogenases (Harris and Waters, 1976; Fersht, 1985), the chloroplast enzymes contain one cysteine that is directly involved in catalysis, cys-149 (numbered according to the model enzyme *Bacillus stearothermophilus* glyceraldehyde-3-P dehydrogenase) and the near neighbor cys, cys-153, in each subunit. These are located on the same helical segment and cannot disulfide bond to any other cys groups. Besides these two common cys residues, there are three unique cys groups in all higher plant glyceraldehyde-3-P dehydrogenase A and B subunits for which sequence information is available (Fig. 1 *A*). Cys residues are not found in these positions in the glyceraldehyde-3-P dehydrogenases from bacteria, animals, or plant cytosol. Our simulation shows that these 3 cys residues are not scattered randomly on the molecule, but are close to one another and located at the surface (Fig. 2) distal to the active site. The

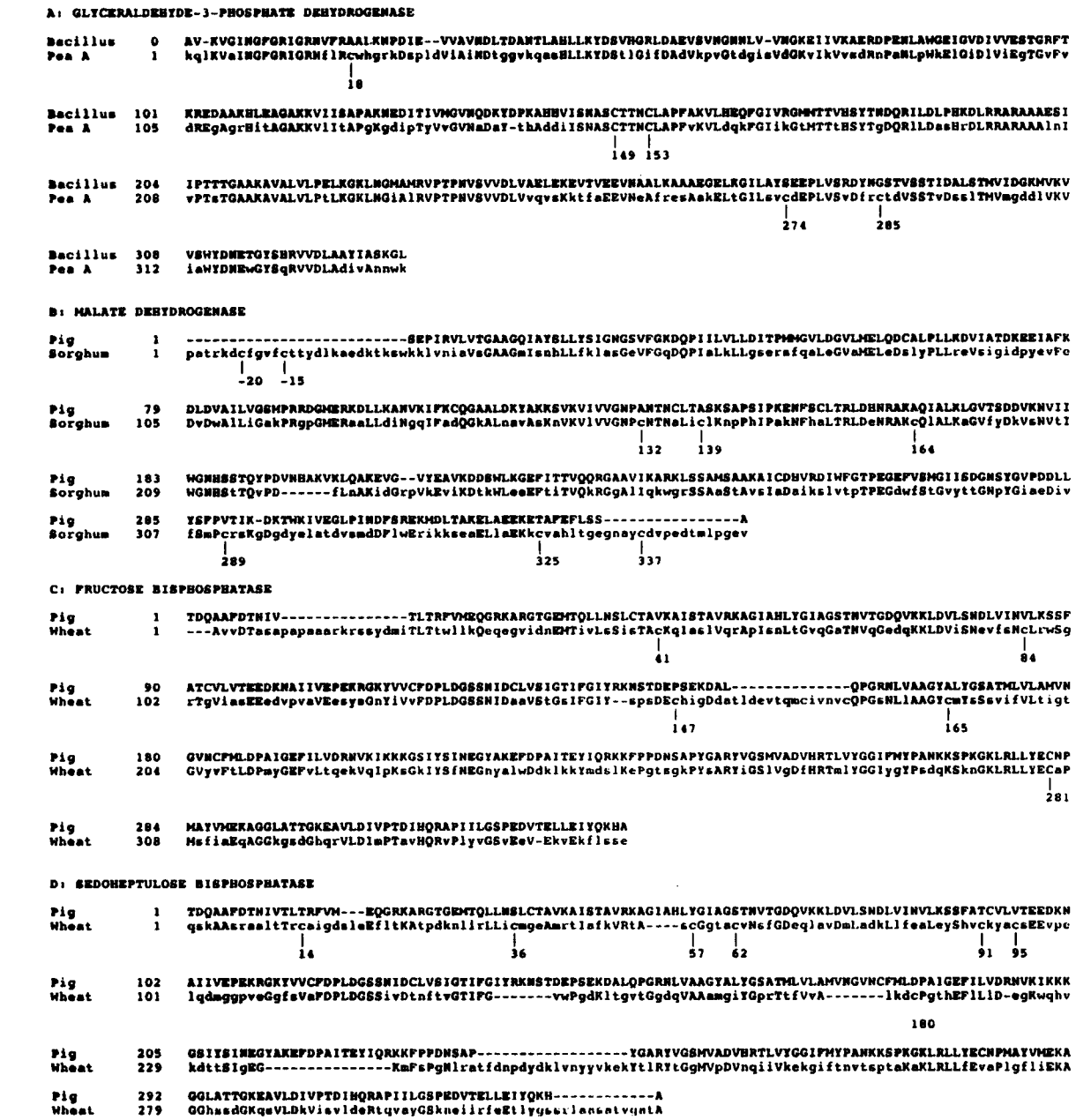

FIGURE 1 Sequence alignments. Cys residues in the chloroplast enzymes are flagged with the number of the corresponding residue in the reference enzyme. Residues identical to the reference sequence residues are in upper case. (*A*) *Bacillus stearothermophilus* glyceraldehyde-3-P dehydrogenase (Biesecker et al., 1977) (*top*) and pea glyceraldehyde-3-P dehydrogenase A (Liaud et al., 1990) (*bottom*). Note that the numbering system for the reference enzyme is the same as that in the Brookhaven Protein Data Bank, which includes some additions and deletions. After primary structure alignment, there is 58% sequence identity between pea chloroplast glyceraldehyde-3-P dehydrogenase subunit A and *Bacillus* glyceraldehyde-3-P dehydrogenase. The 5 cys groups of the A subunits from pea (Liaud et al., 1990), *Arabidopsis thaliana* (Shih et al., 1991) and tobacco (Shih et al., 1986) occupy identical positions after alignment. The A subunit of spinach (Ferri et al., 1990), maize (Quigley et al., 1988, 1989), and *Pinus sylvestris* (Genbank) contains one additional cys residue. All B subunits that have been sequenced, spinach (Brinkmann et al., 1989; Ferri et. al., 1990), pea (Liaud et al., 1990; Brinkmann et al., 1989), tobacco (Shih et al., 1986), and *Arabidopsis* (Shih et al., 1991), have two additional cys residues in the C-terminal extension. (*B*) Pig heart malate dehydrogenase (Birktoft et al., 1989) (*top*) and sorghum chloroplast malate dehydrogenase (Crétin et al., 1990) (*bottom*). (An additional 57 residues for the transit peptide are included by Crétin et al. (1990) in their numbering.) After primary structure alignment, the sequence identity between sorghum chloroplast and pig heart malate dehydrogenase is 44%. Eight unique cys residues are found in all of the chloroplastic malate dehydrogenases sequenced to date including sorghum (Crétin et al., 1990; Luchetta et al., 1990), maize (Metzler et al., 1989), ice plant (Cushman, 1993), and pea (Reng et al., 1993). (*C*) Pig kidney fructose bisphosphatase (Marcus et al., 1982) (*top*), wheat chloroplast fructose bisphosphatase (Raines et al., 1988) (*middle*), and sedoheptulose bisphosphatase (Raines et al., 1992) (*bottom*). After primary structure alignment, the sequence identity between wheat chloroplast and pig kidney fructose bisphosphatase is 45%. There are seven cys residues common to the chloroplastic fructose bisphosphatases from wheat (Raines et al., 1988; Lloyd et al., 1991), spinach (Marcus and Harrsch, 1990), pea (Genbank), rape (Genbank), and *Arabidopsis thaliana* (Horsnell and Raines, 1991), and one additional cys group in the potato enzyme (Kossmann et al., 1992). After primary structure alignment, the sequence identity between wheat chloroplast sedoheptulose bisphosphatase and pig kidney fructose bisphosphatase is 29%.

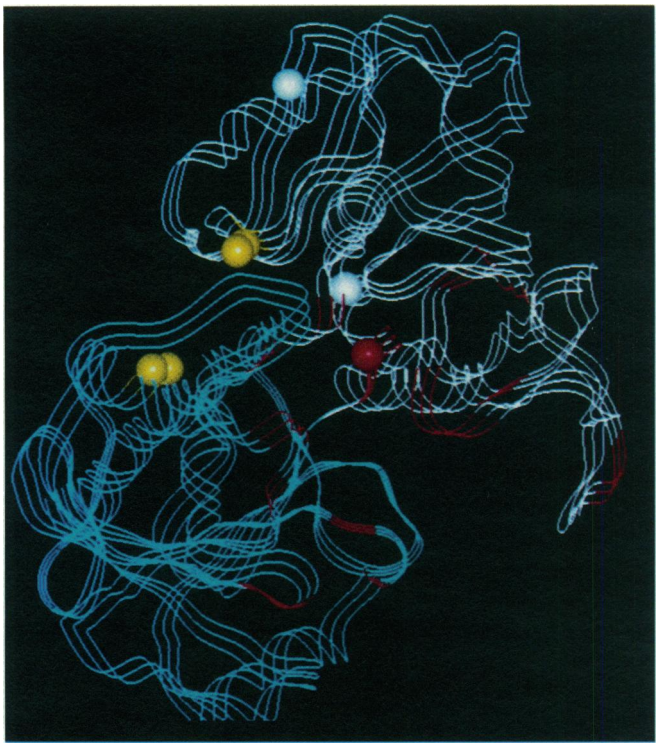

FIGURE 2 Predicted tertiary structure of pea chloroplast glyceraldehyde-3-P dehydrogenase. The corresponding residues on the *Bacillus stearothermophilus* glyceraldehyde-3-P dehydrogenase are replaced by pea chloroplastic cys residues (shown as *spheres*). Active site residues are shown in red. The carbon substrate-binding domain is shown in white, and the nucleotide-binding domain is shown in blue. The distance between cys residues 18 and 285 (shown in *yellow* in CPK representation) is 10.7 Å. Residues are numbered according to the reference enzyme. We used coordinates (Entry 1GD1, version of Oct. 1989) for *Bacillus* glyceraldehyde-3-P dehydrogenase (Skarżyński et al., 1987) obtained from the Protein Data Bank at Brookhaven National Laboratory (Bernstein et al., 1977; Abola et al., 1987). Active site residues are according to Biesecker et al. (1977).

active site of this dehydrogenase consists of a nucleotide-binding region and a carbon substrate-binding region located on two different domains that are connected by a flexible hinge. Catalysis involves movement between the two domains (Skarżyński and Wonacott, 1988). Small changes in the positions of the structural elements opposite the active site should both alter the geometry of the active site and affect the movement of the enzyme: it would appear that movement between the two domains will be restricted if cys-18 and cys-285 form a disulfide bond. This would require that the domains move about 3 Å towards one another, which would change the conformation of the catalytic site. Based on our model, we would expect to find these two residues disulfide-bonded together in the dark (less active) form of the enzyme. Note that the modeling of this structure, and of the malate dehydrogenase structure that follows, is for the active (reduced) form, because the reference structures are non-light-regulated enzymes.

There are two additional cys residues in the C-terminal extension of subunit B. Although the location of the extended C-terminal region of subunit B cannot be predicted because no similar region is present in glyceraldehyde-3-P dehydrogenases for which crystallography data are available, it is not impossible that it could fold back to cys-18, 274, or 285.

Malate dehydrogenase

Malate dehydrogenase, like glyceraldehyde-3-P dehydrogenase, consists of two distinct domains (Hill et al., 1972). One domain contains the nucleotide binding site, and the other contains the carbon substrate binding site. In the closely related lactic dehydrogenase domain movement upon substrate binding has been documented (White et al., 1976). Similar studies of malate dehydrogenase have not yet been reported. There are eight unique cys residues in the chloroplastic malate dehydrogenases that have been sequenced to date, all in identical positions after alignment (Fig. 1 *B*). Cys residues are not found in these positions in other malate dehydrogenases. The chloroplastic enzymes have additional residues at both termini as compared with other malate dehydrogenase enzymes. The N-terminal extension contains two cys residues at positions −15 and −20, and the C-terminal extension contains 1 cys residue at position 337 (numbering according to the model enzyme, pig heart malate dehydrogenase). In malate dehydrogenase, residues 305–330 form an α-helix that runs first along one domain and then along the other domain (Fig. 3). Near this helix is a short helix (residues 131–141) that includes cys residues at positions 132 and 139. Rotation of the two helices would bring cys-325 close enough and into the correct orientation to form a disulfide bond with cys-132 or 139. This would lock the two domains into place and interfere with the conformational changes associated with catalysis.

According to Issakidis et al. (1993), mutation of cys-325 and the two N-terminal cys residues (-15 and -20) of the sorghum malate dehydrogenase produces a fully active, thioredoxin-insensitive enzyme, whereas mutation of the N-terminal cys residues only affects the kinetics of activation (Issakidis et al., 1992). Therefore, they identified cys-325 as one of the cysteines involved directly in activation. Jackson et al. (1992) also implicated cys-325 in light modulation of this enzyme on the basis of computer modeling studies. They suggested that it forms a disulfide bond with cys-337 in the C-terminal extension.

Recently, Reng et al. (1993) constructed a *Pisum sativum* malate dehydrogenase in which four cys residues were mutated to ala: these cys residues were the two cys residues located in the N-terminal extension at positions −15 and −20, cys-164, and cys-337. They found that the quadruple mutant was active in the absence of added reductants, but the triple mutants in which either cys-164 or cys-337 was not mutated were inactive until they were treated with DTT. Therefore, there cannot be a disulfide bond that affects enzyme activity between any two of these four residues. The statement in the abstract of their paper that cys residues 164 and 337 "either alone or in combination with" the cys residues in the N-terminal extension are "responsible for enzyme activation" is not a correct interpretation of their data as given in

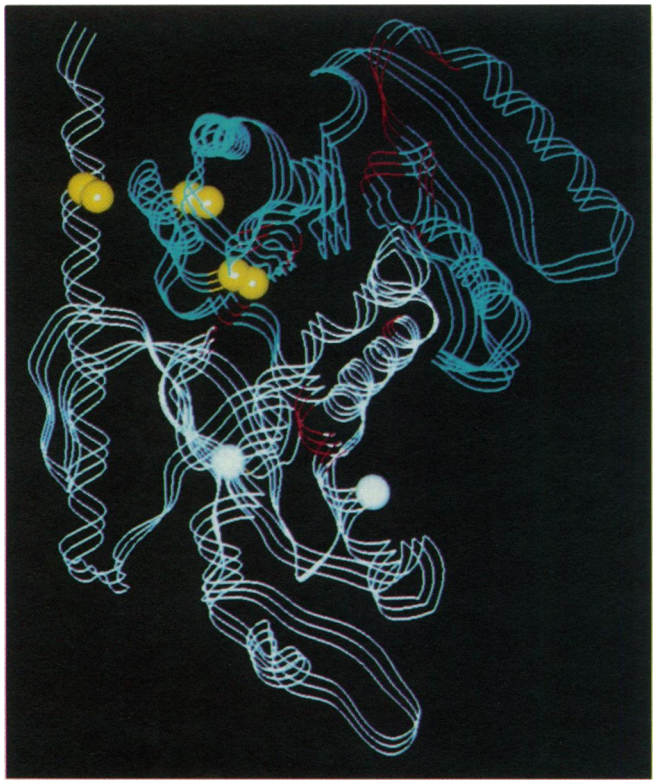

FIGURE 3 Predicted tertiary structure of sorghum chloroplast malate dehydrogenase. The corresponding residues on pig heart malate dehydrogenase are replaced by sorghum chloroplastic cys residues (shown as *spheres*). Active site residues are shown in red. The carbon substrate-binding domain is shown in white, and the nucleotide-binding domain is shown in blue. The cysteines shown in CPK representation in yellow are 325 in the long helix on the left, then clockwise, 139 and 132. The distance between cys residues 325 and 132 is 14.4 Å and between cys residues 325 and 139, 15.0 Å. Residues are numbered according to the reference enzyme. Active site residues according to Birktoft et al. (1989). We used coordinates (Entry 4 MDH, version of Jan. 1990) for pig heart malate dehydrogenase (Birktoft et al., 1987) obtained from the Protein Data Bank at Brookhaven National Laboratory.

the paper. Their data do suggest that there could be two disulfide bonds, one involving cys-164 and the other involving cys-337, that take part in light activation. If cys-164 and cys-337 are involved in disulfide bonds, then their partners must be two other unidentified cys residues. (We note that denaturing gel electrophoresis experiments run in the presence and absence of mercaptoethanol offer no indication of inter subunit disulfide bond(s). See Fig. 6 in Schiebe et al., 1991.) Issakidis et al. (1994) have now produced a quadruple mutant enzyme in which the two N-terminal cysteines and the two C-terminal cysteines (325 and 337) are all mutated. The isolated quadruple mutant malate dehydrogenase is fully active and diamide-sensitive. They nevertheless propose a model in which there are two disulfide bonds, one between the N-terminal cys residues and the other between the C-terminal cys residues.

We predict that a disulfide bridge between cys-325 and either cys-132 or 139 is the crucial disulfide involved in light activation of this enzyme. The presence of additional disulfide(s) could stabilize the dark form of the enzyme and account for the apparently disparate results obtained in the site-directed mutagenesis experiments.

Hatch and Agostino (1992), in studies of the kinetics of activation and reduction of maize malate dehydrogenase, found evidence for reduction of two different disulfides with different redox potentials. One of the disulfides can be reduced by monothiols. Reduction of this disulfide does not affect the activity of the enzyme, but it does increase the rate of activation and presumably, therefore, the rate of reduction of the second disulfide. Genetic truncation of the N-terminus results in a mutant enzyme that is more rapidly activated than the wild-type enzyme (Issakidis et al., 1992). These results suggest that the N-terminal extension, which we cannot model, obstructs the approach of reduced thioredoxin to the regulatory disulfide. Removal of several C-terminal residues by carboxypeptidase increases both the activity of the pea chloroplast enzyme before reductive activation and the rate of activation (Fickenscher and Scheibe, 1988). Removal of the C-terminal extension should render the disulfide between cys-325 and cys-132 or -139 more accessible and increase the rate of activation, as observed.

In the extensively studied bovine pancreatic trypsin inhibitor (BPTI), there are a number of disulfide-containing intermediates that are formed during folding. Rearrangement is the predominant pathway because the rate of formation of native (final) disulfides is too low (Goldenberg, 1992). The multiplicity of surface-localized cys groups on the light-modulated enzymes in the present study might, like the cys groups on BPTI, facilitate reduction of disulfides (light activation) and/or oxidation of dithiols (dark inactivation). Further, a cys in the signal peptide of the pro-form of BPTI, which can be replaced by a tethered cys, enhances thiol-disulfide exchange (Weissman and Kim, 1992). It is tempting to suggest that some or all of the cys residues on the N- and C-terminal extensions peculiar to the light-modulated forms of glyceraldehyde-3-P dehydrogenase and malate dehydrogenase have a similar function.

Fructose bisphosphatase

Modeling the tertiary structures of the chloroplast fructose bisphosphatases shows that the six predictable cys residues (seven for potato) are located in the same region (Fig. 4). Two of these residues, cys-41 and cys-165 (numbered according to pig kidney fructose bisphosphatase), are located in the interior of the protein close enough to one another (7 Å) and in the correct orientation to form a disulfide bond. We suggest that chloroplast fructose bisphosphatase contains an internal disulfide bond that is not involved in reductive activation. In fact, there is evidence for a disulfide bond in the purified protein (see Fig. 1 in Marcus et al., 1987). Cys-281 is close to the active site and inaccessible. Cys-84 and 147 are located on the surface of the protein. Two additional cys residues are contained in a 14- to 17-residue loop that has apparently been inserted between residues 153 and 154 (Marcus et al., 1988) (Fig. 1 *C*). One of the cys residues is adjacent to residue 154 and should be located on the surface.

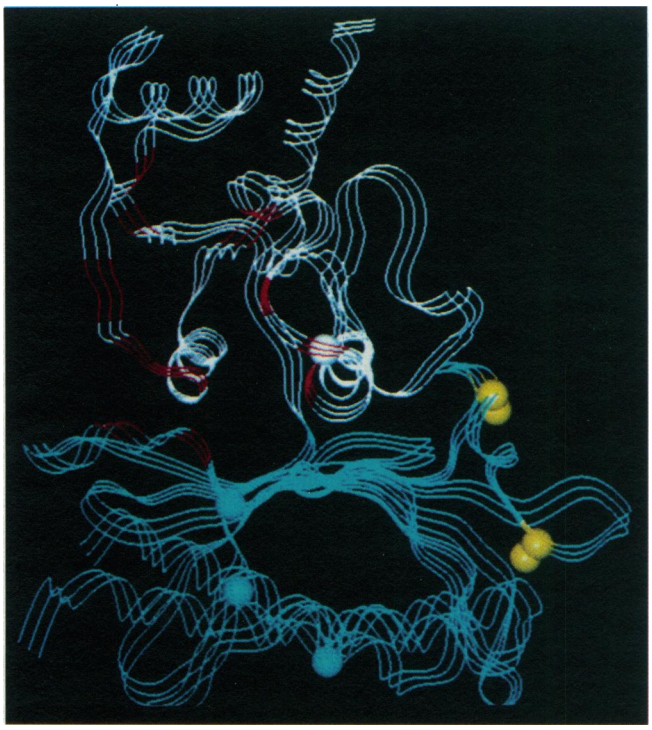

FIGURE 4 Predicted tertiary structure of wheat chloroplast fructose bisphosphatase. The corresponding residues on pig kidney fructose bisphosphatase are replaced by wheat chloroplastic cys residues (shown as *spheres*). Active site residues are shown in red. The AMP-binding domain is shown in blue. Cysteines 147 and 154 are shown in yellow in CPK representation. The chloroplastic enzyme contains an insert of variable length, which is not present in the reference structure and was not modeled. The insert contains two cys residues. One of these is adjacent to residue 154. Therefore, we modeled residue 154 as the cysteine. The distance between residues 147 and 154 is 13.5 Å. Residues are numbered according to the reference enzyme. Active site residues according to (Zhang et al., 1993). We used coordinates (Entry 4FBP, version of April, 1992) for pig kidney fructose bisphosphatase (Ke et al., 1991) obtained from the Protein Data Bank at Brookhaven National Laboratory. This is the AMP-inhibited form of the pig enzyme and should be most similar to the dark, inactive form of the chloroplast fructose bisphosphatase.

The location of the other cys residue in the insertion cannot be predicted because there is no analogous residue in the crystal structure, but it might be close enough to interact with the three surface-located cys residues. Notably, the extended loop is very close to the second domain and is probably in contact with it. Formation of a disulfide bond between cys-147 and the cys adjacent to 154 would affect the conformation of the loop, which might be expected to affect domain-domain interactions.

Like glyceraldehyde-3-P dehydrogenase and malate dehydrogenase, fructose bisphosphatase also consists of two separate domains connected by a single polypeptide hinge (Ke et al., 1989). The active site residues are located on these domains on opposite sides of the active site cleft (Zhang et al., 1993). The cys groups are located distal to the active site residues on the AMP-binding domain (Fig. 4). Apparently, light modulation is mediated through covalent modification of a region that is sensitive to conformational change and that is utilized in other forms for noncovalent allosteric modification. The chloroplastic enzyme is not AMP-sensitive. The mechanisms proposed by Ke et al. (1991) and Liang et al. (1993) for allosteric inhibition of the pig enzyme are probably applicable in modified form to the chloroplast enzyme.

Sedoheptulose bisphosphatase

There are also seven cys residues in the deduced amino acid sequence of wheat chloroplastic sedoheptulose bisphosphatase (Raines et al., 1992) (Fig. 1 *C*). The enzyme shares 29% amino acid identity with pig kidney fructose bisphosphatase and, hence, with the exception of cys-57 and 62 where the corresponding residues are missing, we were able to predict the location of the cys groups in sedoheptulose bisphosphatase on the basis of the tertiary structure of the pig fructose bisphosphatase (Fig. 5). Modeling shows that the cys groups are again located in the AMP-binding domain and at or near the surface of the enzyme. Surprisingly, there is no correspondence in the positions of the seven cys groups in the primary sequences of chloroplastic fructose bisphosphatase and sedoheptulose bisphosphatase, and there is no apparent internal disulfide in the case of sedoheptulose bisphosphatase. Cys-36 and cys-91 are close enough and in the correct orientation to form a disulfide bond. This disulfide would be accessible to thioredoxin. It is significant that disulfide bond formation will be a covalent modification in an

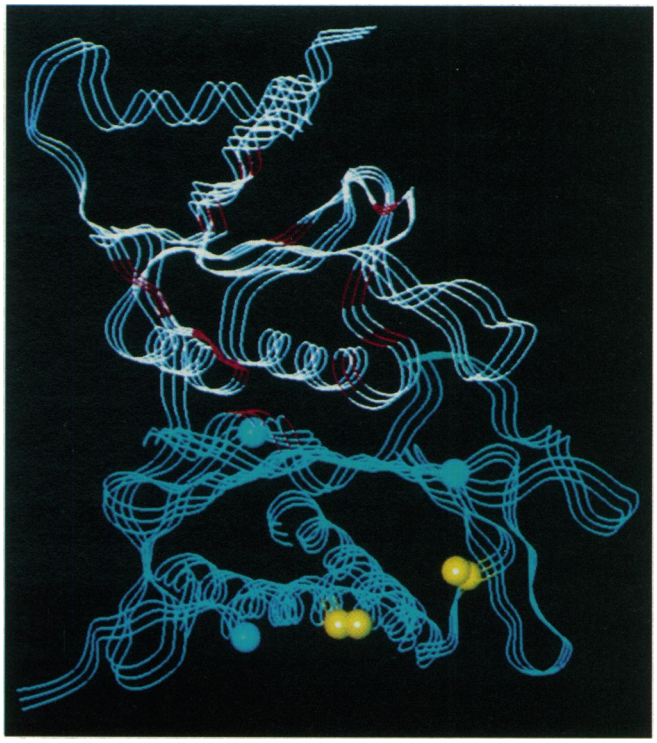

FIGURE 5 Predicted tertiary structure of wheat chloroplast sedoheptulose bisphosphatase. The corresponding residues on pig kidney fructose bisphosphatase are replaced by wheat chloroplastic sedoheptulose bisphosphatase cys residues (shown as *spheres*). Active site residues are shown in red. The AMP-binding domain is shown in blue. The distance between cys residues 36 and 91 (shown in *yellow* in CPK representation) is 11.0 Å.

area known in the closely related fructose bisphosphatases to be involved in allosteric regulation and conformational change.

CONCLUSIONS

Our results indicate that the four light-modulated enzymes we have studied, glyceraldehyde-3-P dehydrogenase, fructose bisphosphatase, sedoheptulose bisphosphatase, and malate dehydrogenase, contain in each case several cys residues located together on the surface of the enzyme distant from the active site. The catalytic activity of each of these enzymes depends on movement in and between two domains connected by a hinge. We have identified disulfide bonds that would potentially lock the enzyme into the inactive dark form in glyceraldehyde-3-P dehydrogenase, malate dehydrogenase, fructose bisphosphatase, and sedoheptulose bisphosphatase. In every case, the resultant cystines would be located distant from the active site and, as required for reduction by thioredoxin, on accessible surfaces. We find no indication of direct interaction with any active site residues. Light modulation of these four enzymes is then apparently allosteric modification, which results from disulfide bond reduction. Modification of enzyme activity by disulfide bond formation has been observed for at least one nonchloroplast enzyme: the Ca^{2+}-dependent form of cardiac troponin C is converted to an active Ca^{2+}-independent form by oxidation of cys residues in vitro (Putkey et al., 1993). Disulfide bond formation is accompanied by a change in conformation.

Notably, there seems to be no correspondence in the positions of the cysteine residues in either the primary or the tertiary structures of the these four light-activated enzymes. These enzymes evolved before the evolution of oxygenic photosynthesis and, presumably, before thioredoxin-dependent light modulation of enzyme activity. The evolution of activity modulation must have occurred independently for each of these enzymes. Apparently, in each case the same mechanistic strategy was used: restriction of domain movement and, hence, of catalysis. But the sites involved in interaction with thioredoxin evolved independently by convergent evolution at least 4 times as, apparently, did light modulation.

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