Structural Basis for a Cofactor-dependent Oxidation Protection and Catalysis of Cyanobacterial Succinic Semialdehyde Dehydrogenase*

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Background: Succinic semialdehyde dehydrogenase from Synechococcus is an essential enzyme in the tricarboxylic acid cycle of cyanobacteria.

Results: Structure of the binary and ternary complex was determined in complex with NADP(H) and/or substrate.

Conclusion: The enzyme forms a distinct reaction intermediate in each complex.

Significance: Structural and functional analysis of the reaction intermediate highlights details of an oxidation-resistance and a reaction mechanism.

Succinic semialdehyde dehydrogenase (SSADH) from cyanobacterium Synechococcus differs from other SSADHs in the γ-aminobutyrate shunt. Synechococcus SSADH (SySSADH) is a TCA cycle enzyme and completes a 2-oxoglutarate dehydrogenase-deficient cyanobacterial TCA cycle through a detour metabolic pathway. SySSADH produces succinate in an NADP⁺-dependent manner with a single cysteine acting as the catalytic residue in the catalytic loop. Crystal structures of SySSADH were determined in their apo form, as a binary complex with NADP⁺ and as a ternary complex with succinic semialdehyde and NADPH, providing details about the catalytic mechanism by revealing a covalent adduct of a cofactor with the catalytic cysteine in the binary complex and a proposed thiohemiacetal intermediate in the ternary complex. Further analyses showed that SySSADH is an oxidation-sensitive enzyme and that the formation of the NADP-cysteine adduct is a kinetically preferred event that protects the catalytic cysteine from H₂O₂-dependent oxidative stress. These structural and functional features of SySSADH provide a molecular basis for cofactor-dependent oxidation protection in 1-Cys SSADH, which is unique relative to other 2-Cys SSADHs employing a redox-dependent formation of a disulfide bridge.

An NAD(P)⁺-dependent succinic semialdehyde dehydrogenase (SSADH²; EC 1.2.1.79) catalyzes the oxidation of succinic semialdehyde (SSA) into its corresponding acid, succinate (Fig. 1A). SSADHs are ubiquitous in a wide range of organisms, including humans, bacteria, plants, and mammals. In eukaryotes, mitochondrial SSADH plays a crucial role in the γ-aminobutyrate shunt (1, 2), where it is responsible for the final reaction in the shunt and thus provides the carbon contained in γ-aminobutyrate as a form of succinate to the ATP-generating tricarboxylic acid (TCA) cycle. Mutation-induced malfunction or a deficiency in SSADH activity has been attributed to an uncommon and heritable neuropharmacological disorder in humans (3). Similar deficiencies in plants cause various developmental and phenotypic changes (4, 5).

Similar to the role of human and plant SSADH in secondary metabolism, bacterial SSADH plays an important role in carbon and nitrogen metabolism (6). Recently, SSADH was recognized to play a novel role in the primary metabolic pathway of cyanobacteria. The TCA cycle in cyanobacteria has long been considered incomplete, mainly due to a lack of 2-oxoglutarate dehydrogenase, an enzyme that catalyzes the conversion of 2-oxoglutarate to succinyl-CoA (7). However, the recent identification of two different genes for 2-oxoglutarate decarboxylase and SSADH from Synechococcus sp. PCC 7002 indicates that the TCA cycle in Synechococcus bypasses the production of succinyl-CoA and produces succinate from 2-oxoglutarate via SSA using these two enzymes (8). Therefore, cyanobacterial 2-oxoglutarate decarboxylase and SSADH act as members of the TCA cycle by replacing 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase.

SSADH belongs to the aldehyde dehydrogenase (ALDH) superfamily (9, 10), and its structure in humans (11) and Escherichia coli (12, 13) has only recently become available. These investigations highlighted a redox-dependent regulation of SSADH activity. Human SSADH (HsSSADH) contains a catalytic loop with two cysteine residues (2-Cys SSADH), including the catalytic cysteine (Fig. 1C), which form a reversible disulfide bridge.
Structure and Function of Cyanobacterial SSADH

A general mechanism of ALDH family, overall structure, and sequence alignment of SySSADH. A, a general mechanism of ALDH activity is presented with a catalytic cysteine and a general base glutamate. B, the binary complex of SySSADH is shown with NADP⁺, which is structurally similar to the apo form. Two monomers in an asymmetric unit are displayed. For clarity, NADP⁺ is shown in only one of the monomers as a space-filling model. Monomeric SySSADH is composed of three domains: the cofactor binding domain (magenta), the catalytic domain (cyan), and the dimerization domain (green). Secondary structural elements are defined in Fig. 1C. C, the amino acid sequences of SySSADH are compared with other members of the family, including MtSSADH for M. tuberculosis SSADH (gene accession no. CAB03694), EcSSADH for E. coli SSADH (gene accession no. NP_417147), AtSSADH for A. thaliana SSADH (gene accession no. NP_178062), and HsSSADH for human SSADH (gene accession no. NP_001071). Note that the catalytic loop is presented with a catalytic cysteine and a general base glutamate.

Given the redox-dependent regulation of HsSSADH, the oxidant-resistant catalytic features of Mycobacterium tuberculosis SSADH (14), and the ATP-dependent catalytic properties of Arabidopsis thaliana SSADH (15), the regulation of SSADH activity is much more complex than initially thought. The different regulatory mechanisms by which these enzymes are controlled remain to be resolved. In particular, a general catalytic mechanism analogous to that of the ALDH family has long been considered (Fig. 1A) (16, 17). Briefly, a catalytic cysteine carries out a nucleophilic attack on the carbonyl carbon of an incoming SSA, forming a thiohemiacetal intermediate. A thioester intermediate is then formed after a hydride transfer from the proposed intermediate to NAD(P)⁺. Subsequently, a water molecule activated by a general base carries out a deacylation to produce succinate as a product. Here, we determined the crystal structure of cyanobacterial SSADH from Synechococcus sp. PCC 7002 (SySSADH) together with a functional analysis. SySSADH is an NADP⁺-dependent enzyme (8) and differs from HsSSADH and EcSSADH in that it contains only one cysteine in the catalytic loop (1-Cys SSADH) that acts as the catalytic residue (Fig. 1C). Three different structures of SySSADH were determined, including a 1.7 Å resolution apo structure, a 1.4 Å resolution binary complex with NADP⁺, and a 1.4 Å resolution ternary complex with SSA and NADPH. Unexpectedly, a covalent adduct of NADP⁺ was discovered with the catalytic cysteine in the binary complex as well as the proposed thiohemiacetal intermediate in the ternary complex, with different conformations of the cofactor in each complex. These results along with kinetic and functional analyses provide molecular insights into the cofactor-dependent oxidation protection and mechanistic features of SySSADH.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—The gene for SySSADH (GenBank™ accession number NC010475.1) (8) was synthesized from Bioneer (Daejeon, Korea) with codon optimization for expression in E. coli. The resulting DNA was restricted by NdeI and XhoI enzymes and subcloned into a modified pET-28a vector (Merck) containing a tobacco etch virus protease cleavage site between the His₅ tag and a multiple cloning site. For structural studies, seleno-L-methionine-substituted, N-terminal His-tagged SySSADH (residues Met-1 to Lys-454) was expressed in E. coli B834 (DE3) methionine auxotroph cells (Merck). Cells were grown at 37°C in minimal medium supplemented with seleno-L-methionine to an A₆₀₀ of 0.7. The cells were then induced for 16 h at 20°C by the addition of...
of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested and sonicated in buffer A (50 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM DTT). The enzyme was purified using immobilized metal affinity chromatography with buffer A plus 500 mM imidazole. After the purified SySSADH was dialyzed against buffer A, the N-terminal His tag was removed by treatment with tobacco etch virus protease for 16 h at 22 °C. SySSADH was then further purified by immobilized metal affinity chromatography and size-exclusion chromatography using a Superdex 200 column (GE Healthcare) equilibrated with buffer A. The enzyme was concentrated to 13 mg ml⁻¹ based on its molar extinction coefficient of 40,910 M⁻¹ cm⁻¹ at 280 nm.

Crystallization—The apo form of seleno-L-methionine-SySSADH was crystallized at 22 °C using the sitting-drop vapor-diffusion method in a crystallization buffer of 0.05 mM potassium phosphate monobasic, 20% (w/v) PEG8000, and 2 mM CaCl₂. Formation of the SySSADH-ligand complex either with NADP⁺ for the binary complex or NADPH and SSA for the ternary complex was achieved by soaking the crystal with the cofactor and/or substrate. For the binary complex with NADP⁺, a pre-grown crystal of SySSADH was soaked for 60 min in a solution of 0.05 mM potassium phosphate monobasic, 20% (w/v) PEG8000, 30% (v/v) ethylene glycol, and 50 mM NADPH. An additional 50 mM SSA was included in the soaking solution for the ternary complex. In both experiments, ethylene glycol was used as a cryoprotectant.

Data Collection and Structure Determination—Multilength anomalous dispersion data for the apo form of seleno-L-methionine-SySSADH were collected to a resolution of 1.7 Å with buffer A. The enzyme was concentrated to 13 mg ml⁻¹ using a Superdex 200 column (GE Healthcare) equilibrated against buffer A, the N-terminal His tag was removed by treatment with tobacco etch virus protease for 16 h at 22 °C. SySSADH was then further purified by immobilized metal affinity chromatography and size-exclusion chromatography using a Superdex 200 column (GE Healthcare) equilibrated with buffer A. The enzyme was concentrated to 13 mg ml⁻¹ based on its molar extinction coefficient of 40,910 M⁻¹ cm⁻¹ at 280 nm.

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Data Collection and Structure Determination—Multilength anomalous dispersion data for the apo form of seleno-L-methionine-SySSADH were collected to a resolution of 1.7 Å on beamline 7A of the Pohang Accelerator Laboratory (Pohang, Korea). Single-length anomalous data were collected at 1.4 Å on beamline 5C at the same facility for the binary and ternary complexes. All data were collected at 100 K and processed using the program HKL2000 (18). The crystals belonged to the space group P2₁2₁2₁, with two monomers per asymmetric unit (Table 1). The structure of SySSADH in its apo form was determined by molecular replacement after failing to locate selenium atoms using single- and multilength anomalous dispersion data. The program PHENIX with an AUTOMR option (19) was used for molecular replacement, with PDB ID 3EFV (43% sequence identity; Center for Structural Genomics of Infectious Diseases) as a search model. Manual model building and refinement were performed using the programs COOT (20) and PHENIX, respectively. After several iterative cycles of manual inspection and refinement, the model was built including Ile-3 to Lys-454 for each monomer. The refined apo form was then used as an initial model to solve the structures of the binary and ternary complexes. Structural refinements were performed including a rigid body refinement followed by simulated annealing and TLS refinement. At the final refinement stage, water molecules, whose refined temperature factors were less than 50 Å², were assigned based on possible hydrogen bonds to the enzyme or other nearby water molecules. Details regarding data and structural refinement are described in Table 1. Structural comparisons and analyses were carried out using the CCP4 suite program (21). Figures were prepared using PyMOL (DeLano, W.L., PyMOL Molecular Graphics System, Palo Alto, CA).

Dehydrogenase Activity Assays—Genes for mutant enzymes were prepared by site-directed mutagenesis using a QuikChange kit (Agilent Technologies) with mutagenic primers. All enzymes, including the WT enzyme, were expressed in E. coli BL21(DE3) strain as an expression host (Agilent Technologies). The N-terminal His-tagged enzymes were purified in the absence of DTT using immobilized metal affinity chromatography and dialyzed against 50 mM HEPES (pH 7.6). Enzyme assays for the steady-state kinetics were performed at 30 °C using a UV-visible spectrophotometer (Jasco). Our standard assay solution contained 100 mM HEPES (pH 7.6), 10 mM DTT, 10 mM CaCl₂, 15 μg (153 nm) of SySSADH enzyme. With the addition of 2.5 mM

**TABLE 1**

| Soaking Condition | Apo | Binary | Ternary |
|-------------------|-----|--------|---------|
| **Soaking Time**  | 60  | 60     |         |
| **Data collection** |     |        |         |
| Space group       | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ |
| Cell dimensions   | a, b, c (Å) | 42.8, 115.3, 179.3 | 42.9, 115.5, 180.1 |
| Wavelength (Å)    | 0.97935 | 0.97948 | 0.97948 |
| Resolution (Å)    | 30.4-1.7 | 15.0-1.4 | 15.0-1.4 |
| Fσ₁ - κFobs (%)   | 20.1 | 20.1 | 20.1 |
| Completeness (%)  | 100 | 100 | 100 |
| Redundancy        | 14.3-1.7 | 13.9-1.4 | 13.9-1.4 |

**Refinement**

| Resolution (Å) | 3.1-1.7 | 3.1-1.4 | 3.1-1.4 |
|----------------|---------|---------|---------|
| No. unique reflections | 19820 | 176393 | 176908 |
| Rmerge (Fσ₁ - κFobs) | 17:3 | 17:3 | 17:3 |
| Average B-factors | 13.8 | 13.8 | 13.8 |
| Bond lengths (Å) | 0.006 | 0.006 | 0.006 |
| Bond angles (°) | 1.1 | 1.1 | 1.1 |
| Ramachandran plot | 97.7 | 97.8 | 97.8 |
| Allowed (%) | 2.2 | 2.2 | 2.2 |
| Disallowed (%) | 0.1 | 0.1 | 0.1 |

* Numbers in parentheses refer to data in the highest resolution shell.
* Rmerge = 100|Ih — <Ih>)/|Ih, where I<sub>obs</sub> is the observed intensity and <I<sub>obs</sub> is the average intensity.
* Rmerge = 100|<I<sub>obs</sub> — k|<I<sub>calc</sub>|/|<I<sub>calc</sub>|
* Rmerge is the same as Rmerge for a selected subset (10%) of the reflections that was not included in prior refinement calculations.
* Ordered residues: the apo structure and the binary complex (Ile-3 to Lys-454 in two monomers) and the ternary complex (Ala-2 to Lys-454 in two monomers).
* Ligand: the apo structure (phosphate ion and ethylene glycol), the binary complex (NADPH and ethylene glycol), and the ternary complex (NADPH, SSA, and ethylene glycol).
* Disallowed residues identified using a program Molprobity (35): the apo structure (Gly-386 in one monomer), the binary and ternary complex (Gly-386 in two monomers).
NADP⁺, the reaction mixture was incubated for 10 min at 30 °C. The reaction was then initiated by the addition of 200 μM SSA. Initial velocity was determined by measuring the linear increase in absorbance at 340 nm for the first 30 s and calculated as the NADPH concentration produced per min. The molar extinction coefficient of NADPH at 340 nm was 6220 M⁻¹ cm⁻¹. Km and Vmax values for NADP⁺ were calculated using SigmaPlot (Systat Software).

Activity Assays under H2O2-dependent Oxidative Stress—To characterize the functional role of the cofactor-cysteine adduct in response to oxidation, we measured the dehydrogenase activity of SySSADH under various levels of H2O2-dependent oxidative stress. To rule out the possible contribution of the reducing agent DTT in this assay, purification of SySSADH was carried out in the absence of DTT as described under “Dehydrogenase activity assays” (above), and its activity was measured using DTT-free standard assay solution unless otherwise specified. EcSSADH (12, 13) was also purified in the absence of DTT as described above.

RESULTS

Overall Structure of ApoSySSADH—SySSADH is a dimeric protein in solution as characterized by size-exclusion chromatography. Consistent with this observation, no higher oligomerization states were evident from the crystallographic symmetry of the P2₁2₁2₁ space group. Therefore, the two monomers in an asymmetric unit represent the functional unit of the dimer and are related by a non-crystallographic 2-fold symmetry with a root mean square deviation of 0.10 Å for 452 Ca atoms from Ile-3 to Lys-454.

The overall structure of monomeric SySSADH is reminiscent of an ALDH fold (22) and similar to the structures of HsSSADH (11) and EcSSADH (12, 13). It is made up of three segments: α/β-fold N- and C-domains for a cofactor binding and a catalytic domain, respectively, and three antiparallel β-strands constituting a dimerization domain (Fig. 1, B and C). Specifically, the N-domain (residues 3–103, 121–230, and 420–444) contains a five-stranded parallel β-sheet (β5–β9) sandwiched between three helices (α1, α6, α7) on one side and four helices (α2, α3, α4, α5) on the other side. The seven-stranded parallel β-sheet (β10–β16) in the C-domain (residues 231–419) is flanked by three helices (α10–α12) and two helices (α8, α9). Juxtaposition of the N- and C-domains generates an active site tunnel between the two domains that is accessible from both ends. The catalytic residues are located in the middle of the tunnel. A nucleophile Cys-262 is located in the catalytic loop between α8 and β11 of the C-domain, and a general base Glu-228 is located in an interdomain-connecting loop between β9 and β10. Dimerization is mediated largely by N-domain α7 and the three antiparallel β-strands (β3, β4, β17) protruding from the N- and C-domains, resulting in a total of 2313 Å² for the buried surface area.

In addition to these structural features, which are also observed in other SSADHs, we identified the binding sites of a phosphate ion and ethylene glycol near the entrance of the tunnel. These sites act as a binding site for cofactor (see below). Specifically, the phosphate ion from the crystallization buffer is located at the N terminus of α6 and the C terminus of β6 in the N-domain. The hydroxyl group and the main chain nitrogen atom of Ser-157 and the side chain amino group of Lys-154 form possible hydrogen bonds within 3.0 Å of the oxygen atoms of the phosphate ion. In addition to these contacts, the positive helix dipole at the N terminus of α6 likely stabilizes the binding of the phosphate ion.

Structure of SySSADH in a Binary Complex with NADP⁺—Structural analysis of the binary complex revealed a covalent linkage between the catalytic residue Cys-262 and the C4 atom on the nicotinamide ring of a cofactor (Fig. 2, A and B). Formation of this adduct occurred when NADP⁺, not NADPH, was present. This was true even when a crystal of SySSADH was soaked with NADPH. NADPH was later discovered to be spontaneously oxidized to NADP⁺ under these experimental conditions (data not shown).

One molecule of the cofactor adduct with Cys-262 was clearly located in each subunit (Figs. 1B and 2A). Overall, the cofactor moiety in the adduct adopted an extended conformation, with the nicotinamide ring and its associated ribose being completely buried in the tunnel. The newly formed covalent linkage to the sulfur atom of Cys-262 was mediated with a pro-R face at C4 of the nicotinamide ring (Fig. 2B), resulting in an sp3 configuration at C4 of the nicotinamide ring, with bond angles of 108° and 109° for the C3–C4–S and C5–C4–S linkages, respectively. The carbonyl amide group of the ring interacts with the

FIGURE 2. The cofactor-cysteine adduct and its binding mode in the binary complex. A, the NADP-cysteine adduct is shown with NADP⁺ (yellow) and Cys-262 (green). The illustration is overlaid with an omitted 2Fo – Fc electron density map contoured at 1.0 σ. B, a different view of the NADP-cysteine adduct is shown with an omitted 2Fo – Fc electron density map (1.0 σ). Atoms in the nicotinamide ring are labeled. During refinement, the distance between the C4 atom of the nicotinamide ring and the sulfur atom of Cys-262 was restricted to 1.9 Å, based on values predicted by quantum mechanical calculations (27) and an observed Fo – Fc electron density map. C, the NADP moiety in the adduct is represented in a stereo view with its binding environment. Residues interacting with NADP⁺ are included along with water molecules (red sphere). See “Structure of SySSADH in a Binary Complex with NADP⁺” for details of the interactions.
in the binary complex, is further stabilized by possible stacking tunnel, relative to its position in the binary complex. The nicotinamide moiety and its associated ribose group. The nicotinamide moiety and its associated ribose group mediates a possible hydrogen bond to the main chain nitrogen atom of Trp-130 and the hydroxyl group of Ser-207. In contrast, a ribose in the adenosine moiety is located near the entrance of the tunnel at a site enclosed by hydrophobic residues including Phe-132, Ile-263, and Val-190 in the N-domain. Specifically, the 2'-phosphate group in the ribose moiety occupies the binding site of the phosphate ion that was identified in the apo form. Almost identical binding modes were observed with Ser-157 and Lys-154. The 3'-hydroxyl group mediates a possible hydrogen bond to the main chain carbonyl oxygen atoms of Met-128 and His-155. The adenine base is inserted into a concave, hydrophobic pocket formed between the α6 and α7 helices with its aromatic ring parallel to the helical axes of those two helices and surrounded by Ala-187 and Val-190 in α6 and Ala-210 and Leu-214 in α7. An additional binding site for ethylene glycol was also identified in the binary complex. It is located in the active site tunnel opposite the cofactor entrance and is located ~4.0 Å from Cys-262 (data not shown).

**Ternary Complex of SySSADH with NADPH and SSA**—The presence of NADPH and SSA facilitated the formation of a ternary complex among SySSADH, the cofactor, and the substrate. Unlike in the binary complex, the cofactor remains as free NADPH, based on the sp^3^ configuration of the C4 atom in the nicotinamide ring (Fig. 3, A and B). SSA forms a covalent linkage with the catalytic residue Cys-262 (Fig. 3C), resulting in a tetrahedral, thiohemiacetal intermediate (Fig. 1A).

NADPH in the ternary complex exhibits a bent conformation, with a large change in the dihedral angle of the pyrophosphate group. The nicotinamide moiety and its associated ribose is no longer in the active site tunnel but has moved away from the active site Cys-262 by about 5 Å toward the entrance of the tunnel, relative to its position in the binary complex. The nicotinamide ring, which is nearly at the position occupied by ribose in the binary complex, is further stabilized by possible stacking interactions with Phe-361. The carbonyl amide group of the nicotinamide ring interacts with nearby residues, including the side chains of Asn-131, Gln-136, and Glu-228 as well as the main chain carbonyl oxygen atoms of Glu-228 and Leu-229. Several interactions are associated with ribose in the nicotinamide moiety and pyrophosphate group. Glu-359 maintains its interaction with the 2'-hydroxyl group of ribose, and water-mediated hydrogen bonds are predominant with the pyrophosphate group. Moreover, the 2'- and 3'-hydroxyl groups of ribose replace the binding site of ethylene glycol that was conserved in both the apo form and the binary complex. Except for these alterations, the binding environment for the adenosine moiety is essentially identical with that of the binary complex.

In the active site, the substrate SSA replaced the ethylene glycol that was identified in the binary complex. The bound SSA forms a covalent adduct with Cys-262 with angles of 109°, 119°, and 105° for the O-C4-S, O-C4-C3, and S-C4-C3 bonds, respectively (Fig. 3, C and D). The SSA-cysteine adduct thus represents a thiohemiacetal intermediate in catalysis. The refence of the trigonal C4 atom in SSA was subject to a nucleophilic attack by Cys-262 to produce an (R)-thiohemiacetal intermediate. The possible positive charge of the O4 atom in the intermediate can be neutralized by a hydrogen bond within 3.1 Å of the carbonyl amide group of NADPH and the side chain of Asn-131. The carboxyl group of SSA is within 3.0 Å of Trp-135, Arg-139, Ser-419, and several water molecules (Fig. 3D). In addition to these hydrophilic residues, the binding site is enclosed by hydrophobic residues including Phe-312, Ile-263, and Phe-425.

**Structural Comparison of Different Ligation States**—No significant changes were noted (within a root mean square deviation of 0.10 Å for 452 Ca atoms) in the overall conformations of the three structures characterized in this study. Noticeable alterations occurring along the catalytic reaction were localized within the cofactor and active site residues. Specifically, different binding modes were observed for the cofactor in the binary and ternary complexes. These were associated with a 5 Å positional shift of the nicotinamide moiety along the tunnel and a change of 85° in the dihedral angle of C4-C5-O–P_N (Fig. 4, A–C).

The catalytic loop (Asn-257 to Lys-266), which contains the nucleophile Cys-262, remains in the same position in each of
the three ligation states. However, significant changes were observed in the position of the side chain of Glu-228. In the apo form, the side chain of Glu-228 is aligned along the wall of the tunnel (Fig. 4A). Although its orientation remains in this position in the binary complex, an intrusion of the cofactor into the tunnel results in interactions between Glu-228 and the carbonyl amide group of the nicotinamide ring (Fig. 4B). The orientation of Glu-228 in the ternary complex differs from those in the apo form and binary complex and is now in the active site tunnel only 3.3 Å from the C4 atom of the thiohemiacetal intermediate, maintaining its interaction with NADPH (Fig. 4C). The side chain of Arg-139 takes on a small-scale change from an interaction with the carbonyl amide group of the nicotinamide ring in the binary complex to an interaction with the carboxyl group of SSA in the ternary complex.

**Functional Analysis of the Active Site Residues**—Preliminary experiments revealed several unusual aspects in the steady-state kinetics of SySSADH (Fig. 5, A–F). In addition to the divalent metal- and DTT-dependent reaction activation, the most unexpected observations were large deviations in the initial velocity that were dependent on the order of the addition of substrate into the assay solution (Fig. 5A). The SySSADH enzyme preincubated with NADP$^+$ showed a linear increase in absorbance after the addition of substrate SSA. Unless, the reaction progress profile exhibited a significant lag, rendering it impractical to reliably measure the initial velocity. Therefore, a standard assay solution was prepared containing DTT, CaCl$_2$, and the SySSADH enzyme. The reaction was initiated by adding 200 μM SSA (see below) after 10 min of incubation with NADP$^+$. A $K_m$ of 439 μM and $V_{\text{max}}$ of 46 μM min$^{-1}$ for NADP$^+$ were successfully measured with 200 μM SSA using the standard assay (Fig. 5E). Those kinetic parameters were not reliably determined for SSA, mainly due to a non-hyperbolic change in initial velocity as a function of the SSA concentration (Fig. 5F). However, the $K_m$ of SSA was estimated to be far less than 50 μM. The catalytic activities of the WT and various mutant enzymes were measured using 200 μM SSA and 2.5 mM NADP$^+$.

The functional roles of various residues in the active site were evaluated (Fig. 3D and 6A). Consistent with the proposed catalytic role of Cys-262 as a nucleophile and Glu-228 as a general base, mutant enzymes such as C262A, E228A, and E228Q were essentially nonfunctional. The substrate SSA binding region is also crucial for enzyme activity. Mutation of residues that interact with the O4 atom or the carboxyl group of SSA abolished
enzyme activity, as observed with mutants N131A, N131D, and S419A. The F425A mutant was also inactive, suggesting that Phe-425 plays an important role in substrate binding. Some mutants, including F132A, W135A, R139A, and I263A, exhibited an activity of about 10–30% that of the WT enzyme, indicating a contribution of these SSA binding residues to the overall enzyme activity. The R139K mutant exhibited an activity up to 80% that of the WT enzyme, further suggesting the significance of a positively charged residue in the binding of the carboxyl group of SSA.

Formation of an NADP-cysteine Adduct in Solution—To validate the presence of a cofactor-cysteine adduct in solution, spectrophotometric measurements were performed in which an equimolar (16 μM SySSADH and NADP) solution of SySSADH and NADP was incubated in a standard assay solution. Absorbance was measured from 300 to 400 nm, which corresponds to the absorbance of the proposed adduct (17, 23). Such an absorption occurred only in the co-presence of SySSADH and NADP+ (Fig. 7A) and disappeared when the WT enzyme was replaced with the C262A mutant (Fig. 7B), thereby validating the formation of an adduct with Cys-262.

Cofactor-dependent Oxidation Protection in SySSADH—Previous structural and functional analyses of 2-Cys HsSSADH showed that HsSSADH is inactivated by H2O2-dependent oxidative stress. Its activity is recovered by adding DTT, which suggests that a nucleophilic cysteine remains protected under oxidizing conditions via the formation of a disulfide bridge with
the second cysteine in the catalytic loop, and that the reduced cysteine is required for activity (11). Due to the lack of a second cysteine, such a mechanism is unavailable in SySSADH as well as in other members of the 1-Cys SSADH family. This leaves the question of whether SySSADH activity is affected by oxidation and how the nucleophilic cysteine of SySSADH can be protected in oxidizing conditions. Given the crystallographic and spectrophotometric identification of the cofactor-cysteine adduct and the linearity of the initial velocity after preincubation with NADP+, we hypothesized that the formation of a cofactor-cysteine adduct is kinetically preferred and could play a role in oxidation protection of the catalytic cysteine.

Five independent assays for SySSADH activity were performed under H2O2-dependent oxidative stress with 50–1000 μM H2O2 and variations of DTT and NADP+ (Fig. 6B). To rule out the possible contribution of a reducing agent DTT in this assay, SySSADH was purified in the absence of DTT, and its resulting activity was also measured in a DTT-free standard assay solution unless otherwise specified. The results were then compared with those obtained in the absence of H2O2. From the first set of experiments (the blue columns), SySSADH was found to be sensitive to H2O2-dependent oxidation. The enzyme activity at 50 μM H2O2 was sharply reduced to 31% that of the H2O2-free enzyme and further decreased to 3% at 1000 μM H2O2. Oxidation effects were nearly eliminated by the co-presence of 10 mM DTT at the onset of the reaction (the red columns), indicating that the reduction in activity of the first set is attributable to H2O2-dependent oxidation. The addition of DTT at 60 min after the H2O2 treatment (the green columns) was not able to fully recover the enzyme activity. However, >70% of the original SySSADH activity was maintained with 50–250 μM H2O2 with a further drop of activity to 40% at 1000 μM H2O2. Comparable or even higher enzyme activity was observed in the fourth set of experiments (the lavender columns) in which SySSADH was preincubated for 10 min with 2.5 mM NADP+ followed by H2O2 treatment for 60 min. In particular, if SySSADH was preincubated in the presence of both NADP+ and DTT, its activity was fully protected even after treatment with 1000 μM H2O2 (the magenta columns). This suggests that under reducing conditions, the catalytic cysteine binds preferentially to a cofactor and remains in a fully active form. This analysis indicates that although SySSADH activity is subject to oxidation, the formation of a cofactor-cysteine adduct plays a key role in protecting the catalytic Cys-262 from oxidation. Furthermore, the adduct is readily dissociable in the presence of a SSA substrate.

Redox-dependent Activity Regulation in a 2-Cys EcSSADH—Previous functional studies of 2-Cys EcSSADH from two independent groups provided contradictory results regarding the presence of a redox-dependent regulatory mechanism in EcSSADH (12, 13). Therefore, the current study addresses whether redox-dependent regulation is present in a 2-Cys EcSSADH and how the enzyme responds to a cofactor. For this assay, EcSSADH was purified in the absence of DTT. First, using our standard assay solution containing 10 mM DTT, EcSSADH was found to require a preincubation with NADP+ to yield a linear initial velocity and a catalytically competent reaction. This was the case unless the enzyme exhibited a lag phase with lower values of initial velocity (inset in Fig. 6C), which suggests the formation of a cofactor-cysteine adduct in the reduced enzyme.

The activity of EcSSADH was measured under various oxidizing and reducing conditions (Fig. 6C). In assay A, a residual activity (5.4 μM min⁻¹) was measured in the absence of DTT and H2O2 after a 10-min incubation with NADP+, suggesting that the purified enzyme contains some fraction of catalytically competent cysteine in its reduced form. Significant variations were observed under oxidizing conditions with 1000 μM H2O2, particularly in the presence of 10 mM DTT at 0, 60, or 70 min into the reaction. In the co-presence of H2O2 and DTT at 0 min (assay B), an EcSSADH activity of 9.7 μM min⁻¹ was measured, which is higher than a residual activity. However, the presence of DTT at 60 min in a solution with preexisting H2O2 (assay C) showed a large increase in EcSSADH activity to 20.3 μM min⁻¹, whereas a marginal activity of 1.7 μM min⁻¹ was observed with an addition of DTT at 70 min in assay D. These large variations in activity are readily explained if the catalytic cysteine remains in its oxidized form in the presence of H2O2, maintained by the formation of a disulfide bond with the second cysteine in the catalytic loop, as observed with 2-Cys HsSSADH (11). Under these conditions, the addition of DTT at 60 min (assay C) reduces the disulfide bond, resulting in a catalytically competent cysteine. However, the functionally inactive, oxidized form of the catalytic cysteine remains dominant in assay D. The presence of both reduced and oxidized cysteine in assay B, due to the co-presence of DTT and H2O2, likely resulted in the enzyme activities between assays C and D.

In contrast, under the reducing conditions presented by the co-presence of NADP+ and DTT, enzyme activity increased significantly, and the enzyme became resistant to oxidative stress. A maximal activity of 39.6 μM min⁻¹ (assay E) was observed after 10 min of incubation. This is 2-fold higher than the activity observed after oxidation and subsequent reduction as in assay C. Further treatment with H2O2 for 60 min under these conditions resulted in an enzyme activity of 30.9 μM min⁻¹ (assay F). However, differences in activity between assays E and F are not due to H2O2-dependent oxidative stress but by some uncharacterized effects caused by the 60-min incubation. A similar reduction of activity was observed in assay G, in which H2O2 was not included in the assay solution but the reaction mixture was incubated for an additional 60 min. In principle, the activities in assays C and E (20.3 and 39.6 μM min⁻¹, respectively) should be comparable as the catalytic cysteine remains in its functionally active form in both cases. The apparently lower activity of assay C is likely due to the catalytically active cysteine being in its reduced form, which is responsible for the residual activity (5.4 μM min⁻¹) observed in assay A. This leaves the cysteine subject to oxidation in assay C. A reduction of activity by as much as 13.8 μM min⁻¹, caused by an unspecified mechanism, was associated with the 60-min incubation time in assay H. Considering a total reduction of 19.2 μM min⁻¹, the enzyme activities in assays C and E are clearly equivalent.

These results strongly support the hypothesis that the catalytic cysteine in 2-Cys EcSSADH exists in an oxidized form as a disulfide bridge under oxidizing conditions. The disulfide bond...
is then fully switched into a catalytically active, reduced form under reducing conditions. Furthermore, once reduced, the catalytic cysteine preferentially forms an NADP-cysteine adduct if NADP⁺ is present. The resulting adduct becomes resistant to H₂O₂-dependent oxidative stress. Therefore, 2-Cys EcSSADH appears to be equipped with two different levels of redox-dependent regulation: disulfide bond formation in the oxidized state and the formation of an NADP-cysteine adduct in the reduced state.

DISCUSSION

From a structural perspective, SySSADH is highly homologous with members of the ALDH family (10). A DALI search (24) revealed that ALDH structures are similar to SySSADH with Z-scores of 14.3–58.6 and root mean square deviations of 1.1–4.4 Å for Cα atoms. However, these similarities were confined within the monomeric structure. Unlike the tetrameric structure exhibited by SSADHs from humans and E. coli (11–13), SySSADH is a dimeric protein that is also characteristic of some members of the ALDH family (10). Extensive reports detailing the structural and functional features of the ALDH family are available (16, 25). Therefore, this section focuses on comparisons of SySSADH with other SSADHs.

From a functional perspective, SySSADH has two unique features relative to other SSADHs: NADP⁺-dependent activity and the presence of only one cysteine residue in the catalytic loop. Sequence conservation of the phosphate-binding site and its accompanying structural features result in a preference for NADP⁺ over NAD⁺ in SySSADH (Fig. 8A). In particular, structural and sequence comparisons have indicated that the residue equivalent to Ser-157 in SySSADH is likely a key determinant of cofactor specificity for NAD⁺ or NADP⁺.

The single cysteine residue, a nucleophilic Cys-262, in the catalytic loop represents another major difference from other 2-Cys SSADHs. This apparently minor variation exerts significant effects on enzyme properties including the static conformation for the catalytic loop, the binding mode of the cofactor, and oxidation protection. Note that most members of the ALDH family contain one cysteine in the catalytic loop and that only 68 of the 1000 members of the family have a catalytic loop with two cysteine residues (data not shown). The catalytic loop (Asn-257 to Lys-266) in SySSADH adopts the same orientation throughout the reaction process (Fig. 4, A–C), leaving the active site tunnel in an opened state. The SySSADH catalytic loop is essentially identical, within a root mean square deviation of 0.3–0.4 Å for 10 Cα atoms, with that of HsSSADH in its reduced form (PDB code 2W8O) (11) and with that of reduced EcSSADH in its complex with NADP⁺ (PDB code 3JZ4) (13). Because the nicotinamide moiety of the cofactor is bound in the tunnel, the conformation state of the active site tunnel affects the binding mode of the cofactor. In fact, the nicotinamide moiety in the SySSADH binary complex occupies the innermost position in the tunnel (Figs. 2A and 8B). In the ternary complex, the nicotinamide moves back along the tunnel, mainly due to the binding of SSA to Cys-262 (Fig. 4C). The resulting location of the cofactor is identical to that in reduced EcSSADH (13). These observations are similar to previous characterizations of the ALDH family in which the nicotinamide half of the cofactor exhibits two different binding mode conformations, “hydride transfer” and “hydrolysis” (26), as the reaction proceeds.

The identification of a covalent adduct between NADP⁺ and Cys-262 in the binary complex of SySSADH is not an isolated
example. Such an adduct was predicted by quantum mechanical calculations (27) and then characterized in two different structures from the ALDH family (17, 23, 28). However, to date, the functional role of the covalent adduct has remained elusive. The results of the current study suggest that the adduct serves to recruit a cofactor in the vicinity of the catalytic nucleophile for catalysis, particularly for hydride transfer. Perhaps more importantly, the adduct provides a novel means of protection against oxidative stress. SySSADH was shown to be an oxidation-sensitive enzyme (Fig. 6B). Oxidation should occur at the nucleophile cysteine in the catalytic loop. Unlike 2-Cys HsSSADH, 1-Cys SySSADH lacks a second cysteine and is, therefore, unable to protect its highly reactive nucleophilic cysteine residue. Instead, SySSADH holds the catalytic loop static, therefore, unable to protect its highly reactive nucleophilic cysteine residue. The formation of a cofactor-cysteine adduct was first reported in 2-Cys EcSSADH (Fig. 6C). This cofactor-dependent oxidation protection is likely applicable to other 1-Cys SSADHs. Moreover, the 1-Cys SSADH from *M. tuberculosis* (Fig. 1C) also exhibited a pronounced lag phase when the enzyme was included as the final component in the reaction mixture (14), which suggests the presence of a cofactor-cysteine adduct. Our analysis further suggests that 2-Cys EcSSADH has two different mechanisms for redox-dependent regulation. In particular, a cofactor-cysteine adduct is formed under reducing conditions (Fig. 6C). Taken together, the formation of a cofactor-cysteine adduct as a means of protecting the catalytic cysteine from possible oxidation is most likely common in members of the SSADH family and ALDH superfamily (17, 23). This unique mechanism differs from the redox regulation of tyrosine phosphatase 1B, when the thiolate of the catalytic cysteine forms a covalent bond with the main chain nitrogen of a nearby residue (32).

In this study we determined crystal structures of an NADP⁺-dependent SySSADH that is involved in the TCA cycle in cyanobacterial *Synechococcus* sp. PCC 7002. The apo structure as well as a binary complex with NADP⁺ and a ternary complex with NADPH and SSA, revealed a structural basis for the formation of a cofactor-cysteine adduct and a thiohemiacetal intermediate, thereby providing details about the catalytic mechanism. In particular, the mechanistic details of SySSADH are similar to those in Fig. 9 and the recently updated mechanism of the ALDH family (23). The current study provides additional insights including the preferred binding of NADP⁺ over SSA, the nucleophilic attack of Cys-262 on the re-face of a trigonal C4 atom in SSA, and the formation of an (R)-thiohemiacetal intermediate. Further kinetic and functional analyses elucidated the functional properties of active site residues and revealed the preferential binding of NADP⁺ to the catalytic cysteine. The formation of a cofactor-cysteine adduct was hypothesized to be a novel means of protecting the catalytic cysteine from oxidation and could be applicable to other members of the SSADH family.

In the middle of revising the manuscript, we note the recent publication of Zheng et al. (33) describing the crystal structure and activity of SSADH Ynel from *Salmonella typhimurium*. The PDB coordinates of Ynel, which were deposited in September of 2008, were used as a starting model of SySSADH in the current study. The structure of Ynel and its complex with NADP⁺ is essentially identical with that of SySSADH, but the NAD⁺ in the binary complex is present in a free form. Through the activity assays, the putative active site residues and mechanism were proposed.

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