A Novel DNA Modification by Sulfur: DndA Is a NifS-like Cysteine Desulfurase Capable of Assembling DndC as an Iron−Sulfur Cluster Protein in *Streptomyces lividans*†

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ABSTRACT: A novel DNA modification system by sulfur (S) in *Streptomyces lividans* 66 was reported to be encoded by a cluster of five genes designated *dndA*−*E* [Zhou, X., He, X., Liang, J., Li, A., Xu, T., Kieser, T., Helmann, J. D., and Deng, Z. (2005) *Mol. Microbiol. 57*, 1428−1438]. The *dndA* gene was cloned and the protein product expressed in *Escherichia coli*, purified to homogeneity, and characterized as a homodimeric protein of ca. 91 kDa. Purified DndA has a yellow color and UV−visible spectra characteristic of a pyridoxal phosphate-containing enzyme and was proven to be a cysteine desulfurase able to catalyze removal of elemental S atoms from L-cysteine to produce L-alanine with substrate specificity similar to that of *E. coli* IscS. DndC was also purified to homogeneity and found to contain a 4Fe-4S cluster by spectral analysis and have obvious ATP pyrophosphatase activity. DndA could catalyze iron−sulfur cluster assembly by activation of apo-Fe DndC protein prepared by removal of its iron−sulfur cluster using α,α′-dipyridyl. A mutated DndA, with serine substituted for cysteine at position 327, which was confirmed to have lost its corresponding cysteine desulfurase activity, also lost its ability to reactivate the apo-Fe DndC. The likely involvement of an interaction between DndA and DndC in the biochemical pathway for the unusual site-specific DNA modification in *S. lividans* 66 is discussed.

Cysteine desulfurase (EC 2.8.1.7) is a pyridoxal 5′-phosphate (PLP)[1] dependent homodimeric enzyme that catalyzes the formation of L-alanine and elemental S by using L-cysteine as substrate. The enzymatic activity was first described for the NifS protein from a diazotrophic bacterium, *Azotobacter vinelandi*. The NifS protein is involved in nitrogenase iron−sulfur cluster formation (*1*). NifS-like proteins, called IscS, were later observed in *A. vinelandii* (*2*). IscS has been found not only in diazotrophs but also in nondiazotrophic microorganisms. NifS homologues are highly conserved among bacteria and eukaryotes and are required for the viability of organisms such as *Escherichia coli* (*3*, *4*), *Salmonella enterica* serovar Typhimurium (*5*), *Bacillus subtilis* (*6*), *Synechocystis* sp. (*7*), *Helicobacter pylori* (*8*), *Saccharomyces cerevisiae* (*9*), and *Arabidopsis thaliana* (*10*).

Most organisms have more than one copy of a *nifS* homologue. *E. coli* contains three genes with sequence homology to *A. vinelandii* nifS, which were named *iscS*, *csdA*, and *sufS* (*csdB*). The deduced IscS protein is 40% identical to *A. vinelandii* NifS, but SufS and CsdA are only 24% identical to NifS (*11*). These enzymes are classified into two groups, based on the sequence similarity of a motif in the active site that contains a conserved cysteine (*4*). By this criterion, *E. coli* IscS is a group I enzyme, whereas SufS and CsdA are members of group II. Genetic experiments have generally revealed important or essential functions for *nifS* homologues. Disruption of the *E. coli* iscS gene results in multiple auxotrophies and severely reduced activity of iron−sulfur cluster enzymes (*12*). Although deletion of *suf* causes no observable growth defects in *E. coli*, such mutants are clearly more sensitive to iron starvation than *isc* mutants. The *sufS* gene is part of the *suf* operon, which is thought to contribute to iron−sulfur cluster formation under conditions of stress (*13*). In addition, the auxotrophies associated with deletion of the entire *isc* cluster in *E. coli* could be corrected by overexpression of the *suf* operon, suggesting that the *suf* and *isc* genes have overlapping functions in iron−sulfur protein biosynthesis.

A specific cysteine residue essential for enzymatic activity has been identified by site-directed mutagenesis. The activity of *A. vinelandii* NifS is inhibited by thiol-alkylating agents (*1*). The cysteine residue acts as a nucleophile to attack the sulfhydryl group of cysteine, resulting in the formation of an enzyme-bound cysteine persulfide intermediate (*14*). The persulfide S is subsequently incorporated into the biosynthetic pathways of S-containing cofactors such as molybdopterin, thiamin, biotin, and lipoic acid, as well as iron−sulfur clusters in proteins and thionucleosides in *E. coli* tRNAs, including 4-thiouridine and 5-methylaminomethyl-2-thiou-

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[1] Abbreviations: ORF, open reading frame; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PLP, pyridoxal 5′-phosphate; dnd, DNA degradation; apo-Fe DndC, DndC devoid of any iron or iron−sulfur clusters; kDa, kilodalton; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol.

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ridine (*15*, *16*). Synthesis of 4-thiouridine requires IscS, which mobilizes S via an L-cysteine desulfurase activity, and ThiI, a protein with adenylation and sulfur transferase activities. IscS and ThiI alone suffice to generate 4-thiouridine. IscS converts cysteine into alanine and forms a persulfide group on an active site cysteine residue, and the S is transferred to ThiI for further transfer to tRNA to generate 4-thiouridine (*17*−*20*).

Iron−sulfur proteins are ubiquitous in bacteria, archaea, and eukaryotes. In most iron−sulfur proteins the clusters function as electron transfer groups, but in others they have other functions such as contributing to catalytic activity, maintenance of protein structure, or regulation of activity (*21*). Iron−sulfur cluster assembly is important for the de novo synthesis of a broad spectrum of proteins and also the repair of certain iron−sulfur proteins that are damaged during oxidative stress. So far, an understanding of the mechanism for iron−sulfur protein assembly has mainly been gained from in vitro experiments. Formation of the iron−sulfur cluster is initiated by the abstraction of S from cysteine in a reaction catalyzed by cysteine desulfurases such as bacterial IscS, NifS, SufS, or yeast Nfs1p (*1*, *9*, *14*). The S atom derived from L-cysteine is transferred directly to a scaffold protein such as IscU and NifU for the assembly of iron−sulfur clusters. Several groups have obtained evidence of complexation and persulfide transfer between IscS and IscU (*22*−*24*). The preformed cluster can subsequently be transferred from IscU to an iron−sulfur apoprotein. Furthermore, some experiments showed that NifS, cysteine, and iron could be used in vitro to reconstitute iron−sulfur clusters of several different iron−sulfur proteins, including the nitrogenase apo-Fe protein (*25*), regulatory proteins apo-FNR (*26*) and apo-SoxR (*27*) from *E. coli*, and apo-biotin synthase from *Bacillus sphaericus* (*28*). A NifS-like protein from the cyanobacterium *Synechocystis* PCC 6803 could be used to produce holoferredoxin from apoferredoxin (*29*). Similar in vitro experiments have shown that IscS is also effective in iron−sulfur cluster formation. Cysteine desulfurase encoded by the gene *iscS* of *E. coli* could directly convert the nitric oxide-modified ferredoxin dinitrosyl iron complex to the ferredoxin [2Fe-2S] cluster in the presence of L-cysteine in vitro (*30*). IscS from *E. coli* could be used to reconstitute the iron−sulfur cluster of dihydroxy acid dehydratase (*3*). IscS can also reconstitute the iron−sulfur cluster of 3′-phosphoadenosine−phosphosulfate−sulfonucleotide reductase (*31*). Although the exact mechanism of S incorporation into iron−sulfur clusters remains unclear, cysteine desulfurase is proposed to play a general role in the formation of iron−sulfur clusters.

In a previous study, we reported identification and characterization of a *dnd* gene cluster for a novel DNA S modification in *Streptomyces lividans* 66 (*32*, *33*). The entire gene cluster (*dnd*) involved in this modification was localized on an 8 kb DNA fragment. A gene cluster (designated *dndA*−*E*) encoding five open reading frames (ORFs) was identified by sequence analysis. *dnd*A was predicted to encode a protein of 397 amino acids with a molecular mass of 43 kDa and close sequence resemblance to *E. coli* IscS (a cysteine desulfurase), and *dndC* was deduced to encode a protein of 498 amino acids with a molecular mass of 57 kDa, with homology to subunit 2 of the sulfate adenylate transferase in the PAPS reductase family of proteins. In order to identify functions for DndA and DndC, and to characterize their possible interactions, both proteins were expressed in *E. coli* and purified to homogeneity. DndA was first characterized as a homodimeric protein containing pyridoxal 5′-phosphate as a coenzyme, and confirmed to catalyze the removal of elemental S atoms from L-cysteine to produce L-alanine, and was subsequently shown to be able to directly activate apo-Fe DndC for its reconstitution as a fully functional [4Fe-4S] cluster protein (DndC) with unambiguously demonstrated ATP pyrophosphatase activity.

MATERIALS AND METHODS

Materials. Escherichia coli strain BL21(DE3) pLysE and plasmid pET-15b were purchased from Novagen (Darmstadt, Germany). Enzymes for DNA manipulation were purchased from New England Biolabs (Beverly, MA), Roche Molecular Biochemicals (Mannheim, Germany), or Takara Shuzo (Kyoto, Japan). Synthetic oligonucleotides were obtained from Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China). Molecular mass markers for gel filtration and columns of HiTrap chelating and Superose 12 (10/30) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Centricon Plus-20 (PL-10) centrifugal filter devices were from Millipore (Bedford, MA). L-Cysteine, L-selenocystine, pyridoxal 5′-phosphate (PLP), and ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2$] were purchased from Sigma-Aldrich. L-Selenocysteine was prepared from L-selenocystine as previously described (*34*). The EnzCheck pyrophosphate assay kit was from Molecular Probes Inc. (Eugene, OR). Coomassie blue protein assay reagent was from Bio-Rad. All other reagents were of analytical grade.

Cloning and Expression of the dndA Gene. The coding region of the *S. lividans* DndA protein was amplified by PCR with pHZ1904 as a template (pHZ1904 harboring the whole *dnd* gene cluster). The following two oligonucleotide primers were used: upstream primer, 5′-GGAATTCCATATGACG-TACGGGACGTGCAG-3′containing an *Nde*I recognition site (underlined) with the ATG start codon; downstream primer, 5′-CGGGATCCTCAATAGCTCGGCTTCAGCTTC-3′ containing a *Bam*HI recognition site (underlined) with the TGA stop codon. The *Nde*I/*Bam*HI-digested PCR product was purified and ligated to the expression vector pET15b (Novagen) to yield pHZ882 containing a six-histidine tag attached to the N terminus of the DndA polypeptide. Plasmid DNA from a positive clone was sequenced and subsequently introduced by transformation into *E. coli* BL21(DE3) pLysE competent cells. The bacteria were grown in 500 mL of Luria−Bertani medium containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol at 30 °C to an A_{600} of 0.8. The *dndA* gene was expressed by induction at 30 °C for 3 h with 0.5 mM isopropyl 1-thio-β-D-galactoside (IPTG). The bacteria were harvested by centrifugation at 5000*g* for 5 min at 4 °C. Cell pellets were resuspended in 15 mL of buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl).

Purification of the DndA Protein. Resuspended bacteria were subjected to three complete cycles of freeze−thawing and then disrupted by sonication. The soluble protein from the cell lysate was collected in the supernatant after centrifugation at 15000*g* for 30 min at 4 °C. The crude extract was applied to a 5 mL HiTrap chelating column (Amersham

Pharmacia Biotech) charged with nickel and preequilibrated with buffer A. The column was subsequently washed thoroughly with buffer A, and the protein was eluted with a linear gradient of buffer B (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 500 mM imidazole). Fractions were analyzed by SDS−PAGE, and those containing His-tagged DndA were pooled and concentrated to 0.5 mL in Centricon Plus-20 (PL-10) centrifugal filter devices (Millipore). The sample was then applied to a Superose 12 10/30 column (Amersham Pharmacia Biotech) equilibrated with buffer A using an ÄKTA fast protein liquid chromatography system (Amersham Pharmacia Biotech). Proteins were eluted with the same equilibration buffer with a 0.5 mL/min flow rate. Fractions containing DndA protein were pooled. The purity of purified DndA protein was greater than 95% judging from its electrophoresis on a 12% polyacrylamide gel containing SDS followed by staining with Coomassie blue. The protein concentration was determined with the Bradford protein assay kit (Bio-Rad) using bovine serum albumin as a standard. The final preparation of the enzyme was stored frozen at −30 °C in the buffer supplemented with 0.02 mM pyridoxal 5′-phosphate until use.

Site-Directed Mutagenesis. The mutants with cysteine residues mutated to serine were prepared by overlap extension PCR. To construct the C327S mutant, two PCRs, with pHZ1904 as the template, were performed with primer pairs DndA-1 (5′-GGAATTCCATATGACGTACGGGACGTG-CAG-3′) plus DndA-2 (5′-GCGCTGGTGGACGCCGAGC-3′) and DndA-3 (5′-GCTCGGCGTCCACCAGCGC-3′) plus DndA-4 (5′-CGGGATCCTCAATAGCTCGGCTTCAGCT-TC-3′). These reactions produced 3′ and 5′ fragments of *dndA*, respectively, whose sequences overlapped by 19 bp at the mutation. By combining the two PCR products as templates and using the *dndA* N-terminal and C-terminal primers, the fused coding sequence of the C327S variant was amplified by PCR. This PCR product was digested with *Nde*I and *Bam*HI, ligated into expression vector pET15b, and then sequenced. A clone with the sequence for the desired C327S mutation was chosen and introduced into *E. coli* BL21(DE3) pLysE. The recombinant cells were used for overproduction and purification of the C327S mutant enzyme.

Preparation of the DndC Protein. The coding region of the *S. lividans* DndC protein was amplified by PCR with pHZ1904 as a template. Two primers were designed to contain an *Nde*I restriction site in one primer and a *Bam*HI site in the other. The sequences of the primers are as follows: DndC-l, 5′-GGAATTCCATATGAGCACCCCCAAG-GC-3′, and DndC-2, 5′-CGGGATCCTTATGCAGGTG-CATCGGTG-3′. The *Nde*I/*Bam*HI-digested PCR product was purified and ligated to the expression vector pRSET-B (Invitrogen) to yield pJTU76 containing a six-histidine tag attached to the C terminus of the DndC polypeptide. pJTU76 was introduced into *E. coli* BL21(DE3) pLysE. The recombinant bacteria were used for overproduction and purification of DndC protein as described above for DndA protein.

Gel Filtration. The native molecular masses of DndA and DndC were determined by gel filtration with a Superose 12 10/30 column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl buffer using an ÄKTA fast protein liquid chromatography system (FPLC). The column had previously been calibrated with the following gel filtration molecular mass markers (Amersham Pharmacia Biotech, Uppsala, Sweden): catalase (232 kDa), aldolase amylase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa).

Assay for Cysteine Desulfurase Activity. DndA desulfurase activity was measured as sulfide production using L-cysteine and L-cystine as substrate as previously described (*35*). Reactions were carried out at 37 °C in glass culture tubes capped with rubber septa stoppers (Waters). The assay was carried out in a 0.8 mL reaction mixture containing 50 mM Tris-HCl (pH 8.0), 0.02 mM PLP, and 5 mM dithiothreitol (DTT) and various concentrations of DndA. The reactions were initiated by the addition of 2.5 mM L-cysteine (Sigma). After a 20 min incubation at 37 °C, the reaction was stopped by adding 100 μL of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 N HCl and 100 μL of 30 mM $FeCl_3$ in 1.2 N HCl. After further incubation in the dark for 30 min, the absorption of methylene blue was measured at 650 nm, and the sulfide concentration was determined on the basis of a standard Na_2S curve.

L-Cystine, L-cysteinesulfinic acid, L-selenocysteine, and L-selenocystine were tested as substrates in a different way. Cysteine desulfinase was assayed by determination of alanine formed from the above substrates with alanine dehydrogenase (*4*). A standard reaction mixture containing 5 mM L-selenocysteine, 5 mM DTT, 0.02 mM PLP, 2.5 mM NAD^+, 120 mM Tris-HCl buffer (pH 8.0), and enzyme in a final volume of 0.5 mL was incubated at 37 °C. A molar turbidity coefficient of NADH at 340 nm, $6.22 \times 10^6\ M^{-1}\cdot cm^{-1}$, was used. Specific activity was expressed as units per milligram of protein, with 1 unit of enzyme defined as the amount that catalyzed the formation of 1 nmol of the product in 1 min.

Assay for ATP Pyrophosphatase Activity. ATP pyrophosphatase activity was monitored by using the EnzCheck pyrophosphate assay kit (Molecular Probes). In this assay, PP_i is converted to P_i by inorganic pyrophosphatase. The substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) is converted enzymatically by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. Enzymatic conversion of MESG results in a shift in absorbance maximum from 330 nm for the substrate to 360 nm for the product. One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 nmol of the product (PP_i)/min. Specific activity was expressed as units per milligram of protein.

In Vitro Reconstitution of the Iron−Sulfur Center. The assembly of the iron−sulfur clusters to the apo-Fe DndC protein was performed under anaerobic conditions (nitrogen atmosphere), as previously described (*25*). To prepare apo-Fe DndC, purified DndC protein (0.17 μmol) was incubated with 1 μM α,α′-dipyridyl in the presence of 2 mM dithionite in a total volume of 0.6 mL at room temperature for 30 min. Apo-Fe DndC protein was purified from the reaction mixture using an FPLC Sepharose 12 10/30 column equilibrated with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl buffer. The absorption spectrum of this sample was recorded. The sample was then made anaerobic and placed in a sealed cuvette. Apo-Fe DndC (40 nmol) was incubated with 0.3−0.6 nmol of DndA protein in the presence of 2.5 mM DTT and 2 mM $Fe(NH_4)_2(SO_4)_2$ in a total volume of 0.6 mL in 50 mM Tris-HCl, pH 7.4, and 50 mM NaCl. The mixture was incubated at 37 °C for 5 min before 1 mM L-cysteine (Sigma) was added. Reconstituted DndC was separated from the reaction

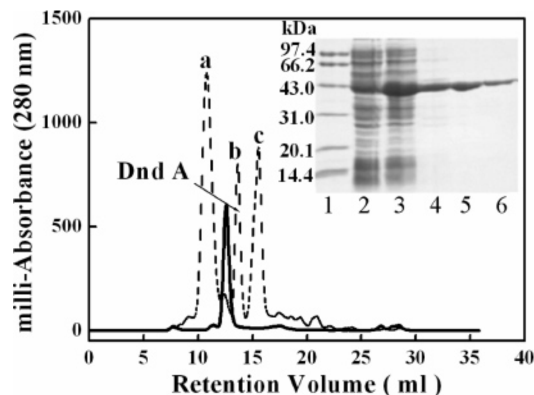

FIGURE 1: Purification of *S. lividans* DndA. DndA after purification on a HiTrap chelating column was loaded on an analytical Superose 12 column and eluted with 20 mM Tris-HCl, pH 8.0, and 50 mM NaCl (solid line). The dashed line shows the elution profile of standard molecular mass proteins on the same column: a, aldolase (158.0 kDa); b, ovalbumin (43.0 kDa); c, ribonuclease A (13.7 kDa). The inset shows SDS−PAGE analysis (Coomassie brilliant blue R-250 stained) of the eluted fractions. Lanes: 1, molecular mass markers (kDa) 97.4, 66.2, 43, 31, 20.1, and 14.4; 2, whole cell lysate after induction; 3, the soluble fraction after sonication; 4 and 5, pooled fractions from HiTrap chelating; 6, pooled fractions from Superose 12.

mixture using an FPLC Sepharose 12 10/30 column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl, pH 7.4, 1 mM DTT, 150 mM NaCl buffer. Iron−sulfur cluster formation was monitored by taking UV−visible absorption spectra in a Perkin-Elmer Lambda 650 spectrophotometer.

Determination of Iron, Acid-Labile Sulfide, and UV−Visible Spectra. The content of iron in DndC protein was determined by atomic absorption spectroscopy in a Varian AA-220 spectrometer using a solution of $Fe(NH_4)_2(SO_4)_2$ in 25 mM HEPES buffer as a standard. Acid-labile sulfide was determined by the method of Beinert (*36*). Optical spectra during DndC reconstitution were recorded on a Perkin-Elmer Lambda 650 two-beam spectrophotometer under anaerobic conditions using a slit width of 2 nm and 1 cm light path. The reference cuvette contained the reaction mixture without DndC protein.

RESULTS

Evidence That DndA Is a Functional Cysteine Desulfurase. The nucleotide sequence of the *dndA* gene incorporated into the expression plasmid (pHZ882) was confirmed to be identical to that registered in GenBank accession number AAZ29040. Induced expression of the gene by addition of IPTG produced ca. 35% of the total soluble cellular protein as DndA, with a molecular mass of 45 kDa as estimated by SDS−PAGE (Figure 1, inset), consistent with the size of the polypeptide deduced from the *dnd*A gene sequence (44984 Da). The purified DndA, using HiTrap chelating and Superose 12, was in the N-terminal His-tagged form, and ca. 15 mg of homogeneous protein could be obtained per liter of culture in a representative purification (Table 1).

The molecular mass of the purified soluble DndA protein was determined to be 91 kDa by gel filtration (Figure 1), implying its presence as a homodimer. The purified DndA protein had a bright yellow color, with an absorbance spectrum (Figure 2) typical of a protein to which the cofactor pyridoxal phosphate is bound. The DndA protein showed an absorption maximum at 420 nm (Figure 2, curve a) at pH 8.0. Reduction by addition of sodium borohydride (1 mM) to the DndA protein solution resulted in disappearance of the absorption band at 420 nm within 2 min, with a concomitant increase in absorbance at 330 nm (Figure 2, curve b). These results are fully consistent with the expected reduction of the imine linkage between the protein and a pyridoxal phosphate cofactor (*37*). The reduced DndA protein was catalytically inactive, and the addition of PLP did not reverse the inactivation.

Table 1: Purification of DndA[a]

step	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)	purification (*x*-fold)
crude extract	1096	2630	2.4	100	1
HiTrap chelating	22	1175	53.4	45	22
Superose 12	15	846	56.5	32	24

[a] Determined with L-selenocysteine as a substrate.

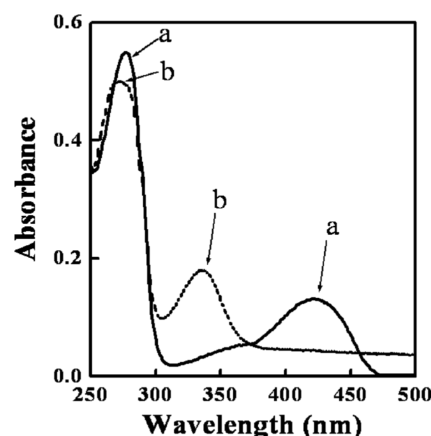

FIGURE 2: Absorption spectra of DndA. Absorption spectra were taken in 20 mM Tris-HCl buffer (pH 8.0) at an enzyme concentration of 0.021 mM. Curves: a, native enzyme; b, 2 min after addition of sodium borohydride (1 mM) to the enzyme solution.

Table 2: Substrate Specificity of DndA

substrate	specific activity (units/mg)	relative activity (%)
L-selenocysteine	56.5 ± 3.2	100
L-cysteine	38.6 ± 2.2	68
L-cysteinesulfinic acid	3.6 ± 0.2	6
L-selenocystine	not detectable	not detectable
L-cystine	not detectable	not detectable

Purified DndA from *S. lividan*s could catalyze removal of a substituent at the β-carbon of L-selenocysteine, L-cysteine, and L-cysteine sulfinic acid to yield L-alanine (Table 2). Although the specific activity of the enzyme toward L-selenocysteine (56.5 units/mg) was lower than that of *E. coli* IscS protein (3100 units/mg) (*11*), the specific activity of the enzyme toward L-cysteine (38.6 units/mg) was comparable to that of *E. coli* IscS (78 units/mg) (*3*) and *A. vinelandii* NifS (89 units/mg) (*1*). The optimal pH value for the removal of selenium from L-selenocysteine was around pH 8.0 in Tris-HCl, in accordance with other cysteine desulfurases. The substrate specificity of the enzyme is summarized in Table 3. The enzyme showed the lowest K_m value and the highest k_{cat} and k_{cat}/K_m values for L-seleno-

Table 3: Substrate Specificity of DndA and Kinetic Constants of the Enzyme Reactions

	K_m (mM)	V_{max} ($nmol{\cdot}min^{-1}{\cdot}mg^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}{\cdot}s^{-1}$)
L-selenocysteine	0.36	56.5	1.4	3.9
L-cysteine	5.1	38.6	0.5	0.098

cysteine, in accordance with the specific activity measurements. The specific activity of the enzyme toward L-cysteinesulfinic acid (3.6 units/mg) was much lower than that of *E. coli* CsdA (20000 units/mg). Thus, the substrate specificity of DndA was similar to that of *E. coli* IscS but different from *E. coli* CsdA and SufS.

Role of Cysteine Residues of DndA. As a cysteine desulfurase, whose representative members (IscS, CsdA, and SufS) could be classified into two groups based on the sequence similarity of a specific motif in the active site that contains a conserved cysteine (*4*), DndA falls into a group (I) with a consensus sequence, ATGSACTS, around a conserved cysteine residue (Cys327) in the C-terminal region. The counterpart cysteine 327 of DndA is highly conserved in many NifS-like proteins and was shown to be crucial for the activity of *A. vinelandii* NifS (*3*). In NifS, this conserved cysteine side chain is thought to bind the S mobilized from free cysteine. Consequently, we replaced this conserved residue with serine by site-directed mutagenesis and determined the cysteine desulfurase activity of the substituted protein. As suspected, the overexpressed and purified serine mutant protein had no cysteine desulfurase activity, consistent with the results obtained using similarly substituted versions of *A. vinelandii* NifS (*3*) and *E. coli* IscS (*14*).

DndA Promotes Iron−Sulfur Cluster Assembly in DndC. We also examined whether DndA could play a role in iron−sulfur cluster assembly, as was shown for *E. coli* IscS and *A. vinelandii* NifS that facilitate formation of iron−sulfur proteins in vitro. As revealed by a BLAST search, DndC in the *dnd* gene cluster has a polycysteine cluster implicating it as a potential iron−sulfur-containing protein. We therefore overproduced and purified His_6-tagged DndC in *E. coli*. UV−visible absorption spectroscopy of the recombinant DndC, eluted from the affinity matrix as a brownish protein, showed a broad absorption band between 350 and 500 nm with a shoulder around 410 nm (Figure 3, curve a), indicating that DndC is indeed an iron−sulfur-containing protein. To gain information regarding the atomic composition of the iron−sulfur cluster in DndC, the content of iron and acid-labile S was determined. Analysis of freshly prepared DndC indicated 4.2 ± 0.05 mol of iron and 4.6 ± 0.4 mol of sulfide per mole of DndC (concentration 0.22 mM per assay, three independent determinations), consistent with the presence of a single [4Fe-4S] cluster per DndC monomer. When apo-Fe DndC was tested, disappearance of its characteristic UV−visible spectrum was observed (Figure 3, curve c). When apo-Fe DndC protein was incubated anaerobically in the presence of L-cysteine, ferrous ammonium sulfate, DTT, and DndA, UV−visible spectra recorded after reconstitution showed an increased peak at 410 nm (Figure 3, curve b), while the 410 nm peak was not observed in a control assay in which heat-inactivated DndA was used instead of DndA. The in vitro assembled DndC and purified DndC exhibited essentially identical spectra, demonstrating successful iron−sulfur center synthesis by the addition of DndA.

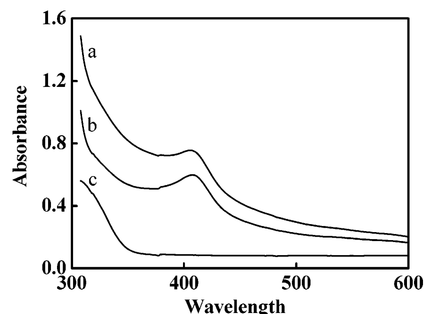

FIGURE 3: Absorption spectra of purified DndC (curve a), restored DndC (curve b) and apo-Fe DndC (curve c). The spectra for purified DndC and apo-Fe DndC were taken in 50 mM Tris-HCl buffer (pH 7.4) with 150 mM sodium chloride at a protein concentration of 0.068 mM. The reconstituted DndC (0.064 mM) was recorded under anaerobic conditions, containing 50 mM Tris-HCl (pH7.4) with 150 mM sodium chloride and 1 mM DTT.

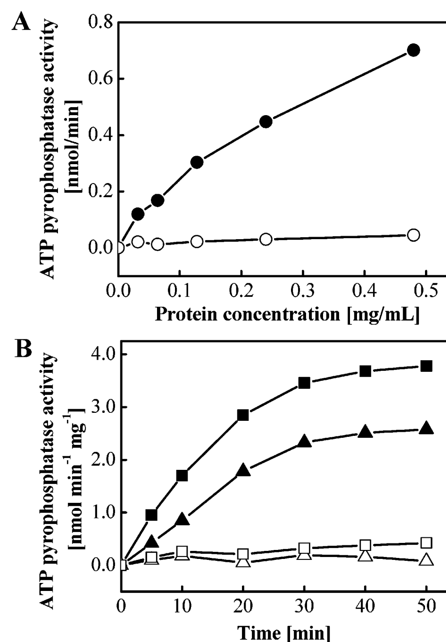

FIGURE 4: ATP pyrophosphatase activity of DndC and reactivation of apo-Fe DndC by DndA. (A) ATP pyrophosphatase activity was measured with various amounts of purified DndC (●) or apo-Fe DndC (○). (B) ATP pyrophosphatase activity of reconstituted DndC. Apo-Fe DndC (40 nmol) was incubated together with various proteins: 0.6 nmol of DndA (■); 0.3 nmol of DndA (▲); 5 nmol of DndA-C327S (□); 5 nmol of heat-inactivated DndA (△).

Reconstitution of the ATP Pyrophosphatase Activity of DndC by DndA. To provide further evidence that *S. lividans* DndA facilitates formation of an iron−sulfur cluster, the specific activity of DndC was examined. Because scrutiny of sequence data revealed that DndC shares a unique “P-loop” motif with the PP_i synthetase family of proteins, we tried to determine its hypothetical ATP pyrophosphatase activity. Indeed, while the specific activity of the native DndC protein as an ATP pyrophosphatase was 4.7 ± 0.2 units/mg of protein, apo-Fe protein, prepared by removing the iron−sulfur cluster using α,α′-dipyridyl chelation, lost its ATP pyrophosphatase activity almost completely (Figure 4A). Spectral data confirmed removal of the iron−sulfur cluster in apo-Fe DndC, correlating its lack of ATP pyrophosphatase activity with the absence of a 4Fe-4S cluster. Subsequently, we investigated the possibility of

restoring enzyme activity by reconstituting DndC by adding recombinant DndA anaerobically to the apo-Fe DndC protein, in the presence of L-cysteine, DTT, and ferrous ammonium sulfate. Interestingly, a rapid increase of ATP pyrophosphatase activity was observed (Figure 4B) under these conditions. Reconstitution of the inactive apo-Fe DndC catalyzed by DndA resulted in 80% recovery of the original activity, and restoration of the enzyme activity was found to depend on the concentration of DndA; a nearly complete restoration could be obtained after the apo-Fe DndC protein was incubated with 0.6 nmol of DndA at 37 °C for 40 min. This increase in ATP pyrophosphatase activity was not found when DndA was replaced by heat-inactivated DndA or the DndA-C327S mutant protein (Figure 4B). In addition, in the absence of ferrous ammonium sulfate or L-cysteine, reconstitution of ATP pyrophosphatase activity was not observed. These results indicated that iron−sulfur centers in DndC are required for its enzymatic activity and that they can be efficiently assembled by DndA.

DISCUSSION

The proposed biochemical pathway (*33*) leading to a novel DNA modification by S involves five putative proteins encoded by the *dnd* gene cluster. An initial comparison with the possible mechanism proposed for *E. coli* tRNA modification by S to synthesize 4-thiouridine, which needs only two enzymes (IscS and ThiI), suggested that the mechanism for DNA modification by S was more complex, as all of the five proteins (DndA−E) seemed to be essential either for direct S incorporation or regulation of the DNA modification (*33*, *38*); additional catalytic steps were likely to be involved. IscS involved in *E. coli* tRNA modification indeed showed extensive homology with DndA of the five proteins for DNA modification. The similar role of DndA with IscS as an L-cysteine desulfurase, which might be essential for the mobilization of S into either DNA or tRNA, and the similar role of DndC with ThiI, sharing a unique adenylation-specific P-loop motif (SGGKDS for DndC and SGGFDS for ThiI), which may function as a sulfate adenylate transferase for activation of the nucleotides to be modified, clearly suggested the existence of common steps in the respective biochemical pathways. The above information, mostly deduced from bioinformatic analysis, needed to be confirmed or revealed by biochemical approaches and prompted us to focus on these two types of enzymes probably shared, at least to certain extent, by both pathways.

In a first experiment, we showed that *S. lividan*s DndA was indeed a PLP-containing homodimer that specifically catalyzes formation of L-alanine and elemental S using L-cysteine as substrate. The fact that the replacement of the normal cysteine residue (Cys327) by a serine in DndA abolished its cysteine desulfurase activity proved the requirement of this cysteine for catalytic function to bind S mobilized from free cysteine, agreeing with the identical role of IscS for tRNA modification in *E. coli* (*18*). DndA could thus be established as an IscS-counterpart protein.

In a second experiment, we also showed that DndC possesses an obvious ATP pyrophosphatase activity, catalyzing hydrolysis of ATP to pyrophosphate (PP_i), whose specific activity (4.7 units/mg) was about 2.5 times of that catalyzed by ThiI (1.9 units/mg; unpublished data). This could be a clear indication that activation of the specific site for modification by adenylation is necessary, resembling the situation for ThiI.

In a further experiment, we demonstrated that the function of DndC could be mediated by formation of an iron−sulfur cluster protein, whose reconstitution was activated by DndA. The exact mechanism for [4Fe-4S] cluster formation stimulated by DndA is not proven, but a likely relevance to its ability to mobilize S for iron−sulfur cluster reconstitution exists. Biosynthesis of iron−sulfur clusters usually needs scaffolding proteins, like NifU or IscU, or NifU(IscU)-like proteins, whose direct counterpart was not obviously found to be encoded by the *dnd* gene cluster. Although five ORFs that are similar to NifS were found in the fully sequenced genome of *Strerptomyces coelicolor* A3(2), a close relative of *S. lividans*, only one (SCO1920) was found to encode a NifU-like protein. Our data could not prove or exclude whether this NifU-like counterpart protein in *S. lividans* would function as a scaffold protein for the assembly and delivery of iron−sulfur clusters to DndC protein. An *E. coli* CSD system contributing to Fe-S biogenesis in vivo, which was known to contain a NifS-like enzyme and a sulfur acceptor protein but no scaffold, was previously reported (*39*). The failure of the other potential five NifS homologues to complement the *dnd*A mutation in *S. lividans* for incorporation of sulfur into its DNA and capability of DndA in catalyzing the activation of DndC in vitro for iron−sulfur cluster formation suggested that the activation of DndC in vivo might be indeed dependent on DndA protein. Iron−sulfur proteins have been reported to play important roles in many fundamental processes of life, such as functioning as electron transfer groups, contributing to catalytic or regulatory activities (*21*), as sulfur donor to provide sulfur for insertion into dethiobiotin (*40*), playing a purely structural role as in *E. coli* endonuclease III (*41*) and MutY (*42*) like Zn in Zn-finger proteins, in which the cluster controls the structure of a protein loop essential for recognition and repair of damaged DNA (*43*). The iron−sulfur-containing DndC protein and its activated formation might be unique and/or specific to the DNA S-modification system. The iron−sulfur-containing feature, and fact that the natural DndC was active only in a dimeric form while apo-Fe DndC was inactive in a polymeric form (data not shown), implied that the functional role of DndC mimics that of ThiI in its adenylation activity but not in other possible structural and/or functional aspects. It was the obvious presence of the shared regional homology with a unique P-loop motif between two proteins, which was known to be important in ThiI for the adenylation of the uridine for its subsequent modification forming 4-thiouridine in the tRNA, that prompted us to propose that the likely formation of a persulfide group between DndA and DndC and activation of the site to be modified by adenylation prior to nucleophilic attack by DndC persulfide for site-specific DNA modification. This resembles the formation of a persulfide group between IscS and ThiI and activation of the uridine by adenylation prior to nucleophilic attack by ThiI persulfide to generate 4-thiouridine in tRNA modification. DndC's additional property as an iron−sulfur cluster as suggested by the detected spectroscopic difference indicates that DndC could also function as a sulfur donor for nucleophilic attack of the site to be modified and that

this is a specific requirement for site-specific modification of DNA, but not RNA.

We propose that adenylation of the site to be modified, and/or the site specificity of the modification into DNA, is dependent on formation of an iron−sulfur cluster for DNA modification, but not for tRNA modification, as mechanistically five enzymes are needed for the former but only two enzymes are needed for the latter. Thus, the cysteine desulfurase DndA is also a central switch for DndC to form as an iron−sulfur cluster protein, which is a prerequisite for performing its ATP pyrophosphatase activity on the site in DNA. The iron−sulfur center of the DndC protein could also have an important structural role for recruitment of DndD and DndE at the modification site. These two proteins, encoded by genes cotranscribed with *dndC*, are believed to be involved in subsequent stages of DNA S-modification. The essential roles of these genes in the proposed biochemical pathway for site-specific DNA modification are under study. Additionally, we are also making an effort to purify DndC protein from a *dnd*A mutant to examine its suspected nonreconstituted activity in order to prove the activating role of DndA for iron−sulfur cluster formation of DndC.

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