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Substrate and Cofactor Range Differences of Two Cysteine Dioxygenases from *Ralstonia eutropha* H16

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Cysteine dioxygenases (Cdos), which catalyze the sulfoxidation of cysteine to cysteine sulfinic acid (CSA), have been extensively studied in eukaryotes because of their roles in several diseases. In contrast, only a few prokaryotic enzymes of this type have been investigated. In *Ralstonia eutropha* H16, two Cdo homologues (CdoA and CdoB) have been identified previously. *In vivo* studies showed that *Escherichia coli* cells expressing CdoA could convert 3-mercaptopropionate (3MP) to 3-sulfinopropionate (3SP), whereas no 3SP could be detected in cells expressing CdoB. The objective of this study was to confirm these findings and to study both enzymes in detail by performing an *in vitro* characterization. The proteins were heterologously expressed and purified to apparent homogeneity by immobilized metal chelate affinity chromatography (IMAC). Subsequent analysis of the enzyme activities revealed striking differences with regard to their substrate ranges and their specificities for the transition metal cofactor, e.g., CdoA catalyzed the sulfoxidation of 3MP to a 3-fold-greater extent than the sulfoxidation of cysteine, whereas CdoB converted only cysteine. Moreover, the dependency of the activities of the Cdos from *R. eutropha* H16 on the metal cofactor in the active center could be demonstrated. The importance of CdoA for the metabolism of the sulfur compounds 3,3′-thiodipropionic acid (TDP) and 3,3′-dithiodipropionic acid (DTDP) by further converting their degradation product, 3MP, was confirmed. Since 3MP can also function as a precursor for polythioester (PTE) synthesis in *R. eutropha* H16, deletion of *cdoA* might enable increased synthesis of PTEs.

Cysteine dioxygenases (Cdos) are thiol-oxygenating enzymes that are well characterized in eukaryotes (1, 2). They catalyze the oxidative conversion of cysteine into cysteine sulfinic acid (CSA) and perform the first step in the catabolism of the highly reactive amino acid cysteine (Fig. 1). Because several neurological disorders, like Alzheimer's and Parkinson's diseases (3) and Hallervorden-Spatz disease (4), have been linked to excess levels of cysteine in plasma or the lack of cerebral cysteine dioxygenase activity, the enzyme is exceedingly interesting for medical research.

Several analyses of the crystal structure were performed, using recombinant Cdos from different mammalian sources (5–7), and revealed an alternative structural motif for coordination of the iron cofactor by Cdos. Whereas most of the nonheme iron proteins coordinate the metal via two histidine residues and a carboxylic acid group (the 2-His–1-carboxylate facial triad), the ferrous iron in Cdos is arranged in a mutually *cis* geometry consisting of three histidine residues (3-His facial triad) (1, 8, 9). The loss of Cdo activity after immobilized metal chelate affinity chromatography (IMAC) purification was reported in several studies (10–12), and the activity could be reconstituted only by addition of exogenous ferrous iron, whereas other transition metals failed to restore the activity. In addition, the inhibition of Cdo activity by chelating agents, like 1,10-phenanthroline or EDTA (13), emphasized the strict dependency of the previously characterized Cdos on ferrous iron. Another unique feature of mammalian Cdo is the formation of a cross-linked Cys-Tyr cofactor that is regulated by cysteine and represents an unusual form of substrate-mediated feed-forward activation of enzyme activity (14). The formation of the Cys-Tyr cofactor requires a transition metal [Fe(II)], as well as oxygen, and it is also strictly dependent on the specific Cdo substrate cysteine (15). In eukaryotes, the mature Cys-Tyr cofactor-containing Cdo and the cofactor-free enzyme exist. Both forms

show catalytic activity, but the cofactor formation leads to a 10-fold increase of Cdo activity and also a prolonged catalytic half-life (14, 16).

Besides eukaryotes, the enzyme was also identified in several eubacteria (11, 15, 17–19). Although the translational products of these homologous genes showed only low overall sequence identity to eukaryotic Cdos, structural and catalytic studies verified that the presence of the enzyme is not restricted to higher organisms (11). In 2009, we identified a Cdo homologue in the Gram-negative bacterium *Variovorax paradoxus* TBEA6 (17). Enzymatic studies showed that the enzyme catalyzed the unusual oxidation of 3-mercaptopropionate (3MP) to yield 3-sulfinopropionate (3SP). Because no oxygenation of other thiols, like cysteine or cysteamine, was observed, the enzyme was referred to as 3MP dioxygenase (Mdo) (17). The conversion of 3MP into 3SP was also shown for the orthologous Mdo in the 3,3′-dithiodipropionic acid (DTDP)-utilizing bacterium *Advenella mimigardefordensis* DPN7[T] and for a Cdo homologue from *Pseudomonas aeruginosa*, which is also able to convert cysteine, but to a much lesser extent (18–20).

FIG 1 Conversion of L-cysteine to L-cysteine sulfinic acid catalyzed by CdoA and CdoB from *R. eutropha* H16 (top) and conversion of 3-mercaptopropionate to 3-sulfinopropionate catalyzed by CdoA (bottom).

The genome sequence of *Ralstonia eutropha* H16 revealed two paralogous *cdo* genes (21), and the amino acid sequences of the translational products (CdoA and CdoB) exhibited significant differences (36% identical amino acids), particularly in the conservation of the typical cupin motifs (22). It was demonstrated that only one of the two Cdos (CdoA) in *R. eutropha* H16 is able to convert 3MP (17) (Fig. 1). In addition, 3MP, 3,3′-thiodipropionic acid (TDP), and DTDP can be used by *R. eutropha* H16 as precursor substrates for the biosynthesis of poly(3-hydroxybutyrate-*co*-3MP) copolymers when a second carbon source is available to support growth (23–25). Enzymatic cleavage of TDP into 3MP and 3-hydroxypropionate (3HP) was previously shown (17, 26), and the cleavage of DTDP into two molecules of 3MP by the dihydrolipoamide dehydrogenase PdhL has also been demonstrated (27).

In contrast to L-cysteine, 3MP does not occur naturally in cells of *R. eutropha* H16. Nevertheless, it is produced in various biological and abiotic reactions and can be found in nanomolar concentrations in natural aquatic environments (28). Thus, it is one of the most frequently detected thiols in these habitats (29, 30). Moreover, 3MP seems to be a central intermediate in sulfur metabolism with, e.g., an important role in the catabolism of dimethylsulfoniopropionate, which is produced by several species of marine phytoplankton, macroalgae, and some angiosperms (31–36). 3MP can also be detected in freshwater habitats and hypolimnetic waters (28, 32, 37). Since 3MP is already toxic to cells in low concentrations, thereby hindering the growth of bacteria like *R. eutropha*, the ability to convert 3MP is advantageous for freshwater bacteria like *R. eutropha* H16, and it could explain the significance of the two different Cdos in the bacterium (38–40).

To get a deeper knowledge of the two Cdos from *R. eutropha* H16, we performed a biochemical characterization of the purified enzymes. Both Cdos were analyzed with regard to their substrate ranges, their quaternary structures, their iron contents, their cofactor dependencies, and the inhibition of their catalytic activities by different thiols and chelating agents.

MATERIALS AND METHODS

Chemicals. Organic thiochemicals of high purity grade were purchased from Acros Organics (Geel, Belgium) or Sigma-Aldrich (Steinheim, Germany). 3SP was synthesized as previously described (18, 41).

Bacterial strains and cultivation conditions. *R. eutropha* H16 was cultivated at 30°C in nutrient broth (NB) under aerobic conditions on a rotary shaker at an agitation of 130 rpm. *Escherichia coli* Rosetta(DE3)(pLysS)(pET23a::*cdoA*), *E. coli* Lemo21(DE3)(pLemo) (pET23a::*cdoA*), *E. coli* Rosetta(DE3)(pLysS)(pET23a::*cdoB*), and *E. coli* Lemo21(DE3)(pLemo)(pET23a::*cdoB*) were cultivated in lysogeny broth (LB) medium. Carbon sources were supplied as filter-sterilized stock solutions, as indicated in the text. For maintenance of plasmids, antibiotics were prepared according to the method of Sambrook et al. (42) and added to the media at the following concentrations: ampicillin, 75 μg/ml; carbenicillin, 75 μg/ml; and chloramphenicol, 34 μg/ml. Heterologous expression of genes under the control of a *lac* promoter was induced by addition of 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Whereas CdoA was found as a highly soluble protein, the CdoB protein was exclusively synthesized in inclusion bodies. Therefore, the cultivation of recombinant *E. coli* Lemo21(DE3)(pLemo)(pET23a::*cdoB*) and *E. coli* Rosetta(DE3)(pLysS)(pET23a::*cdoB*) for heterologous expression of *cdoB* was performed as previously described for a gene from *Streptomyces coelicolor* (11). Due to the long time of cultivation, carbenicillin instead of ampicillin was used for maintaining plasmid stability. All the strains and plasmids used are listed in Table 1.

DNA isolation and manipulation. Chromosomal DNA of *R. eutropha* H16 was isolated according to the method of Marmur (43). Plasmid DNA was isolated from *E. coli* strains using the GeneJet plasmid miniprep kit from Fermentas (St. Leon-Rot, Germany) according to the manufacturer's manual. DNA was digested with restriction endonucleases under conditions described by the manufacturer or according to the method of Sambrook et al. (42). PCRs were carried out in an Omnigene HBTR3CM DNA thermal cycler (Hybaid, Heidelberg, Germany) using Platinum *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany). PCR products were isolated from an agarose gel and purified using the NucleoTrap kit (Machery and Nagel, Düren, Germany) according to the manufacturer's instructions. T4 DNA ligase was purchased from Invitrogen (Karlsruhe, Germany). Primers were synthesized by MWG-Biotech AG (Ebersberg, Germany).

Transfer of DNA. Competent cells of *E. coli* strains were prepared and transformed by the CaCl₂ procedure (42).

DNA sequencing and sequence data analysis. Samples were prepared for sequencing using the BigDye Terminator v3.1 cycle-sequencing kit according to the manufacturer's manual (Applied Biosystems, Darmstadt, Germany). Afterward, the samples were submitted to the Institut für Klinische Chemie und Laboratoriumsmedizin at the Universitätsklinikum Münster, Münster, Germany, for purification of the extension products and sequencing in an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Darmstadt, Germany). BLASTX was used for determination of nucleotide identity (44). For amino acid sequence alignments, Clustal_X (45) and BioEdit (46) were applied. The same sequences were used for the calculation of a phylogenetic tree, employing the neighbor-joining method (47). Besides CdoA and CdoB from *R. eutropha* H16, the well-known eukaryotic Cdos from rat and human were selected, in addition to Cdos from the zebrafish, the nematode *Caenorhabditis elegans*, and the fungi *Arthroderma benhamiae* and *Histoplasma capsulatum*. Representatives of prokaryotic thiol dioxygenases were chosen from *Verminephrobacter eiseniae*, *Variovorax paradoxus*, *Advenella* sp., *Bordetella pertussis*, *Methylibium petroleiphilum*, *Pseudomonas putida*, *Cupriavidus* sp., *Ralstonia* sp., and *Bacillus* sp. due to their sequence similarity to either CdoA or CdoB.

Cloning of *cdoA*ᵣₑ and *cdoB*ᵣₑ. The *R. eutropha cdoA* (*cdoA*ᵣₑ) gene was amplified from total genomic DNA of *R. eutropha* strain H16 by PCR using the high-fidelity enzyme PCR mix (MBI Fermentas, St. Leon-Rot, Germany) and the oligonucleotides *cdoA*(NdeI), 5′-CGAGACACTCCG GAGTCAATCATATG-3, and *cdoA*(XhoI), 5′-CTCGAGTCCTGACTGG GCAACTTCG-3′. The resulting PCR product was isolated from an agarose gel using the NucleoTrap kit (Machery and Nagel, Düren, Germany) and ligated with pJet 2.1/blunt DNA (Promega, Madison, WI, USA.). The *cdoB* gene was amplified using *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany) and the oligonucleotides *cdoB*(NdeI), 5′-CATATG CCGACCGGATCTCCCCTG-3′, and *cdoB*(SalI), 5′-GCAAAACGTCGA CGGCGTCGGC-3′. After purification of the PCR product, the gene was

TABLE 1 Strains and plasmids

Strain/plasmid	Relevant phenotype/genotype[a]	Reference or source[b]
R. eutropha H16	Wild type	62
E. coli TOP10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *rpsL nupG* φ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK endA1*	Invitrogen
E. coli Lemo21(DE3)(pLemo)	*fhuA2* [lon] *ompT gal* (λ *DE3*) [*dcm*] Δ*hsdS*/pLemo(Camʳ) [λ *DE3* = λ *s*BamHIo ΔEcoRI-B *int*::(*lacI*::P*lacUV5*::T7 *gene1*) *i21* Δ*nin5*; pLemo = pACYC184-P*rhaBAD-lysY*]	New England Biolabs Inc.
E. coli Rosetta(DE3)(pLysS)	F⁻ *ompT hsdS*ᵦ(rᵦ⁻ mᵦ⁻) *gal dcm* (DE3)(pLysSRARE) (Camʳ)	Novagen
E. coli TOP10(pET23a(+)::*cdoA*)	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *rpsL nupG* φ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK endA1* Ampʳ (*cdoA* = gene coding for CdoA of *R. eutropha* H16)	This work
E. coli TOP10(pET23a(+)::*cdoB*)	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *rpsL nupG* φ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK endA1* Ampʳ (*cdoB* = gene coding for CdoB of *R. eutropha* H16)	This work
E. coli Rosetta(DE3)(pLysS) (pET23a::*cdoA*)	F⁻ *ompT hsdS*ᵦ(rᵦ⁻ mᵦ⁻) *gal dcm* (DE3)(pLysSRARE) (Camʳ) Ampʳ (*cdoA* = gene coding for CdoA of *R. eutropha* H16)	This work
E. coli Lemo21(DE3)(pLemo)(pET23a::*cdoA*)	*fhuA2* [lon] *ompT gal* (λ *DE3*) [*dcm*] Δ*hsdS*/pLemo(Camʳ) [λ *DE3* = λ *s*BamHIo ΔEcoRI-B *int*::(*lacI*::P*lacUV5*::T7 *gene1*) *i21* Δ*nin5*; pLemo = pACYC184-P*rhaBAD-lysY* Ampʳ; *cdoA* = gene coding for CdoA of *R. eutropha* H16)	This work
E. coli Rosetta(DE3)(pLysS)(pET23a::*cdoB*)	F⁻ *ompT hsdS*ᵦ(rᵦ⁻ mᵦ⁻) *gal dcm* (DE3)(pLysSRARE) (Camʳ) Ampʳ (*cdoB* = gene coding for CdoB of *R. eutropha* H16)	This work
E. coli Lemo21(DE3)(pLemo)(pET23a::*cdoB*)	*fhuA2* [lon] *ompT gal* (λ *DE3*) [*dcm*] Δ*hsdS*/pLemo(Camʳ) [λ *DE3* = λ *s*BamHIo ΔEcoRI-B *int*::(*lacI*::P*lacUV5*::T7 *gene1*) *i21* Δ*nin5*; pLemo = pACYC184-P*rhaBAD-lysY* Ampʳ; *cdoB* = gene coding for CdoB of *R. eutropha* H16)	This work
pCR2.1 TOPO	Ampʳ Kanʳ	TOPO TA cloning kit (Invitrogen)
pJet 2.1/blunt DNA	Ampʳ	Promega
pET23a	Ampʳ	Novagen
pET23a(+)::*cdoA*	Ampʳ (*cdoA* = gene coding for CdoA of *R. eutropha* H16)	This work
pET23a(+)::*cdoB*	Ampʳ (*cdoB* = gene coding for CdoB of *R. eutropha* H16)	This work

[a] Ampʳ, ampicillin resistance; Camʳ, chloramphenicol resistance; Kanʳ, kanamycin resistance. Genotype descriptions are based on those of Bachmann (63).
[b] Sources: Invitrogen, Carlsbad, CA, USA; New England Biolabs Inc., Ipswich, MA, USA; NovagenMerck KGaA, Darmstadt, Germany; Promega Corporation, Madison, WI, USA.

inserted into the cloning vector pCR2.1 TOPO (TOPO TA cloning kit; Invitrogen Corporation, Carlsbad, CA, USA) by topoisomerase reaction. *E. coli* Top10 was transformed with the resulting hybrid plasmids, and transformants were selected on LB agar plates containing IPTG, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and ampicillin. For heterologous expression in the T7 promoter/polymerase-based expression vector pET23a (Novagen, Madison, WI, USA), the *cdo* genes were obtained by restriction of hybrid plasmids pJet2.1/blunt::*cdoA*ᵣₑ and pCR2.1TOPO::*cdoB*ᵣₑ with NdeI and XhoI or NdeI and SalI, respectively. Upon purification from an agarose gel using the NucleoTrap kit (Machery and Nagel, Düren, Germany) the genes were ligated into the expression vector pET23a, which was linearized with the same restriction endonucleases. The ligation products were used for transformation of CaCl₂-competent cells of *E. coli* Top10. After selection of transformants using LB medium containing ampicillin, the hybrid plasmids were isolated, analyzed by sequencing, and used for transformation of *E. coli* Rosetta(DE3)(pLysS) (Novagen, Madison, WI, USA).

Analysis of enzyme assay reaction products by HPLC. Concentrations of 3SP, CSA, and hypotaurine were analyzed by high-performance liquid chromatography (HPLC).

HPLC analysis of 3SP was carried out in a LaChrom Elite HPLC apparatus (VWR-Hitachi International GmbH, Darmstadt, Germany) consisting of a Metacarb 67H advanced C column (Varian, Palo Alto, CA, USA; Bio-Rad Aminex equivalent) and a 22350 VWR-Hitachi column oven. The primary separation mechanism included ligand exchange, ion exclusion, and adsorption. A VWR-Hitachi refractive index (RI) detector (type 2490) with an active flow cell temperature control and automated reference flushing eliminating temperature effects on the RI baseline was used for detection. Aliquots of 20-μl cell-free supernatants, solutions of

organic sulfur compounds, or enzyme assay mixture were injected and eluted with 0.005 N sulfuric acid (H_2SO_4) at a flow rate of 0.8 ml/min and a column temperature of 50°C. Online integration and analysis were done with EZ Chrome Elite software (VWR International GmbH, Darmstadt, Germany). Detection of hypotaurine and CSA was carried out in a Kontron Instrument (Neufahrn, Germany). After derivatization with OPA reagent (48) using a Smartline Autosampler 3900 (Knauer Advanced Scientific Instruments, Berlin, Germany), 20 μl of the reaction mixture was injected onto a Novapack C₁₈ reverse-phase column (Knauer, Berlin, Germany) and monitored fluorimetrically at 330/450 nm (excitation/emission) by using a model 1046A fluorescence detector (Hewlett Packard, Germany). Substances were identified by comparison of their retention times to those of standard organic acids.

Preparation of crude extracts. Cell extracts were obtained either by lysis of the frozen cells using the detergent mixture BugBuster Extraction Reagent (primary amine free; Novagen, Madison, WI, USA) or by mechanical disruption. For this, the cell pellet was resuspended in binding buffer (0.1 M Tris-HCl, 0.5 M NaCl, 20 mM imidazole, pH 7.5) and passed three times through a chilled French press cell (Amicon, Silver Spring, MD, USA). Soluble protein fractions of crude extracts were obtained in the supernatants after 1 h of centrifugation at 100,000 × *g* at 4°C and were subsequently used for enzyme purification.

SDS-polyacrylamide gel electrophoresis. Samples were resuspended in gel loading buffer (0.6% [wt/vol] SDS, 1.25% [vol/vol] β-mercaptoethanol, 0.25 mM EDTA, 10% [vol/vol] glycerol, 0.0001% [wt/vol] bromophenol blue, and 12.5 mM Tris-HCl, pH 6.8). Proteins were denatured by 5 min of incubation at 96°C and separated in 13% (wt/vol) SDS-polyacrylamide gels, as described by Laemmli (49). The proteins were stained with Coomassie brilliant blue R-250 (50). Samples for SDS-polyacryl-

amide gel electrophoresis under nonreducing conditions were prepared by 5-min incubation of protein at 96°C in loading buffer containing 20% sucrose and 0.1% (wt/vol) bromophenol blue.

IMAC. To obtain purified hexahistidine-tagged fusion Cdo, His Spin Trap affinity columns (GE Healthcare, Uppsala, Sweden) were used according to the instructions of the manufacturer with minor modifications. Tris-HCl (0.1 M, pH 7.5) was used as a buffer component instead of sodium phosphate, and for the washing step, a buffer containing 40 mM imidazole was applied. The washing step was repeated up to five times, and the elution step was repeated two times. The purification of Cdo from crude extracts obtained from 2-liter LB cultures was done by using His-Trap FF columns (GE Healthcare, Uppsala, Sweden) with a bed volume of 1 ml. The binding buffer (0.1 M Tris-HCl, 0.5 M NaCl, 20 mM imidazole, pH 7.5) was used for application of the Cdo. The elution of the enzyme was achieved by a stepwise increasing gradient of imidazole. For this, the binding buffer and the elution buffer (0.1 M Tris-HCl, 0.5 M NaCl, 500 mM imidazole, pH 7.5) were mixed with a flow rate of 1 ml/min and a maximum pump pressure of 0.3 MPa. If BugBuster reagent (Novagen, Madison, WI, USA) was used for cell lysis, the obtained soluble cell fraction was diluted 1:1, using the 40 mM imidazole-containing washing buffer, prior to column loading to obtain a solution having the same imidazole concentration as the binding buffer used (20 mM).

Determination of iron content. Determination of the iron content was done with bathophenanthroline disulfonic acid disodium salt (BP-DADS), based on the method of Pierce et al. (9).

Removal of the metal cofactor by dialysis and complementation experiments with different metal ions. After purification of the enzyme by IMAC, the enzyme solution, with a concentration (c) of 2 to 5 mg/ml, was dialyzed against buffer containing 2 mM 1,10-phenanthroline. A dialysis tube (ZelluTrans; molecular weight cutoff [MWCO], 12,000 to 14,000; Carl Roth GmbH & Co. KG, Karlsruhe, Germany) with a flat width of 45 mm and a thickness of 20 μM was used and prepared according to the manufacturer's instructions. After preparation and cooling of the dialysis tube, the enzyme solution was loaded into the tube and transferred to a 1,000-ml beaker that already contained 50 mM Tris-HCl buffer (pH 7.5) and 2 mM 1,10-phenanthroline. The enzyme solution was dialyzed against this buffer for 3 h. Then, the buffer was replaced by pure 50 mM Tris-HCl buffer (pH 7.5), and the enzyme solution was dialyzed against this buffer overnight. Before the dialyzed enzyme solution was used for further experiments, the enzyme was concentrated and transferred to 62 mM MES [2-(N-morpholino)ethanesulfonic acid] buffer (pH 6.5). Before and after the dialysis, the iron content of the enzyme solution was determined. For the complementation experiments, 1.6 to 62.5 mM stock solutions of the divalent metal ions Cu(II), Fe(II), Mg(II), Mn(II), Ni(II), and Zn(II) were prepared in H₂O prepared with a Milli-Q Plus ultrapure water machine (Millipore GmbH, Schwalbach, Germany) and applied in the enzyme assay at a final concentration of 0.05 to 1.5 mM. As a reference value, the enzyme activity was also determined without adding any divalent metal ion.

Enzyme assay. The standard *in vitro* activity of cysteine dioxygenase was assayed by incubating 3 to 14 μg purified Cdo for 30 min at 30°C in the presence of the following components: 0.5 to 10 mM cysteine, 0.5 to 10 mM cysteamine or 0.5 to 5 mM 3MP, 200 to 400 μM (NH₄)₂Fe(SO₄)₂·6H₂O, 12.5 μM bathocuproine disulfonate, and 62 mM MES buffer (pH 6.5). The reaction was stopped by 10 min of incubation at 95°C. Negative controls were performed with denatured protein. The reaction products 3SP and CSA were analyzed by HPLC. Standards of 3SP and CSA were run in HPLC to quantify the enzymatic products. The detection limits were 1 μM and 10 μM, respectively.

SEC. The degree of polymerization of the enzymes was determined by size exclusion chromatography (SEC) on a Superdex 200 column (GE Healthcare, Uppsala, Sweden) after purification of the enzyme by IMAC. As a buffer component, 50 mM sodium phosphate (pH 7.5) was used, with a flow rate of 0.75 ml/min. The maximum pump pressure was set to 1.5 MPa.

Recording of an absorption spectrum. The recording of an absorption spectrum for CdoB was done after purification of the enzyme by IMAC and determination of the protein content. The elution fraction with the highest protein content was used for recording the absorption spectrum. The measurement was done in a UV microcuvette (Plasti-Brand; Brand GmbH & Co. KG, Wertheim, Germany) in a Nicolet Evolution 100 UV-visible light (Vis) spectrophotometer in the scan mode, where the temperature in the photometer was kept constant at 30°C by using an Air-Cooled Single Cell Peltier SPG1A (both from Thermo Fisher Scientific GmbH, Schwerte, Germany).

RESULTS

Characterization of *cdo* homologues in *R. eutropha*. The identification of two *cdo*-homologous genes in the genome sequence of *R. eutropha* H16 (21) was reported in a previous study (17). The gene coding for CdoA (H16_B1863) comprises 618 bp and is located on chromosome 2 (260.54°). The protein consists of 205 amino acids and has a calculated molecular mass of 22.58 kDa with an isoelectric point of 5.8. The paralogous gene, *cdoB* (H16_A1614), is located on chromosome 1 (155.65°) and comprises 576 bp coding for 191 amino acids. The calculated molecular mass of the protein is 20.39 kDa, and its isoelectric point is 7.0.

The phylogenetic relationship of CdoA and CdoB from *R. eutropha* H16 to Cdos from other organisms was analyzed by constructing a phylogenetic tree using various primary sequences of thiol dioxygenases from different kingdoms. As indicated by the resulting tree (see Fig. S1 in the supplemental material), CdoA is very closely related to the putative Cdos from *Cupriavidus necator* N-1 (95% identical amino acids), *Ralstonia pickettii* (94% identical amino acids), and *Cupriavidus taiwanensis* (89% identical amino acids). Comparatively high sequence similarities to the previously described 3MP dioxygenases (Mdo) from *A. mimigardefordensis* DPN7ᵀ (56% identical amino acids) and *V. paradoxus* TBEA6 (55% identical amino acids) were also found (17, 18, 51, 52). The amino acid sequence of CdoA shared 83% identical amino acids with the type I cysteine dioxygenase from *Cupriavidus pinatubonensis* (formerly *R. eutropha*) JMP 134. The latter protein was crystallized by the Institute for Structural Genomics and is available at the MMDB Entrez structure database (accession no. 4QMA) (53).

The translational product of *cdoB* also showed high sequence similarities to different putative Cdos from *C. necator* N-1 (92% identical amino acids) and *C. taiwanensis* (79% identical amino acids) and to a putative Cdo from *B. pertussis* Tohama I (46% identical amino acids), a eukaryotic Cdo from the fungus *H. capsulatum* (32% identical amino acids), and a human Cdo (31% identical amino acids). The sequence similarity of CdoB to members of the 2-mercaptosuccinate dioxygenase (Msdo) subtree amounts to 30% identical amino acids for both putative Cdo-homologous Msdos from the genus *Advenella* and to only 27% identical amino acids of the confirmed Msdo from *V. paradoxus* strain B4 (54, 55). A multiple-sequence alignment illustrates the similarities and differences between all the above-mentioned Cdos (and homologues thereof) in detail (see Fig. S2 in the supplemental material).

Heterologous expression of *cdo* genes from *R. eutropha* H16 and purification of the hexahistidine fusion proteins by IMAC. Both *cdo* genes from *R. eutropha* H16 were heterologously expressed as hexahistidine-tagged fusion proteins using recombinant strains of *E. coli* Lemo21(DE3)(pLemo) or *E. coli* Rosetta(DE3)(pLysS) harboring pET23a::*cdoA* and pET23a::*cdoB*, respectively. Purification of the proteins was achieved by IMAC and resulted in highly purified proteins that were used for further studies
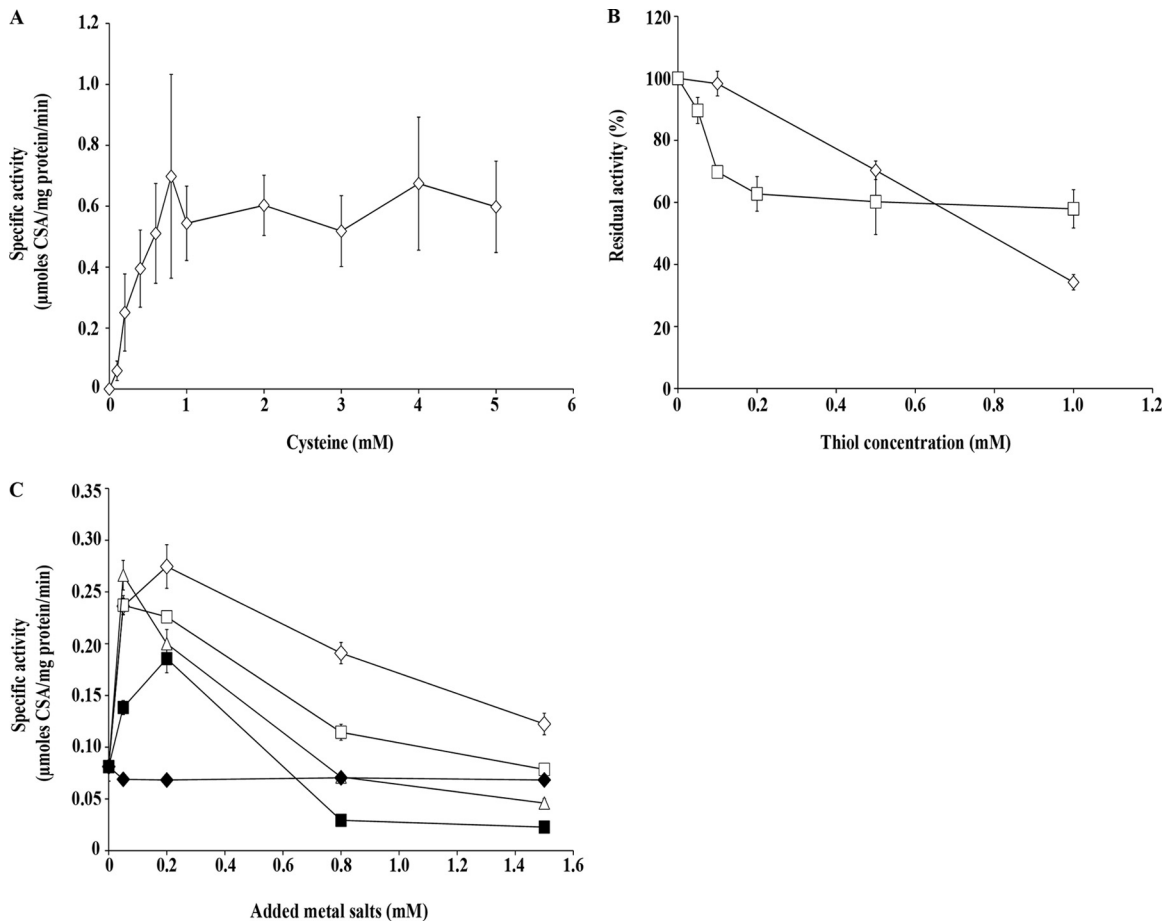
FIG 2 Enzyme activity assay of recombinant CdoB. (A) Dependency of CdoB enzyme activity on various substrate concentrations. Ten micrograms of purified CdoB was incubated in MES buffer (62 mM, pH 6.5) containing various concentrations of cysteine (0.05 to 5 mM), 12.5 μM bathocuproine disulfonic acid disodium salt, and 400 μM $(NH_4)_2Fe(SO_4)_2\cdot6H_2O$ and shaken for 30 min at 500 rpm in a thermoblock (model MHR10; HLC BioTech, Bovenden, Germany). (B) Inhibitory effects of various concentrations of cysteamine ($\diamond$) and 2-mercaptoethanol ($\square$) on CdoB enzyme activity. Purified CdoB (3 to 14 μg) was incubated for 30 min in MES buffer (62 mM, pH 6.5) containing 0.5 mM cysteine, 12.5 μM bathocuproine disulfonic acid disodium salt, 200 μM $(NH_4)_2Fe(SO_4)_2\cdot6H_2O$, and various concentrations (0 to 1 mM) of cysteamine or 2-mercaptoethanol. CdoB activity measured in the absence of an effector was taken as 100%. (C) Influence of various metal salts on enzyme activity of CdoB. Three micrograms of purified CdoB was incubated for 30 min in MES buffer (62 mM, pH 6.5) containing 0.5 mM cysteine and various concentrations (0.05 to 1.5 mM) of the respective metal salt: $FeCl_2$ ($\diamond$), $CuCl_2$ ($\square$), $MnCl_2$ ($\blacklozenge$), $ZnCl_2$ ($\triangle$), and $NiCl_2$ ($\blacksquare$). The results shown represent the averages $\pm$ standard deviations (SD) of three independent biological replicates.

(see Fig. S3a to d in the supplemental material). In contrast to purified CdoA, which was colorless, purified CdoB showed blue color.

Characterization of CdoB. The analysis of purified CdoB by SEC showed an experimental molecular mass of 63.9 $\pm$ 0 kDa (see Fig. S4 in the supplemental material). Since the calculated molecular mass of a hexahistidine-tagged CdoB monomer is 21.3 kDa, the experimental molecular mass shows that CdoB apparently exists as a trimer *in vitro*.

In vitro activity assays verified that the purified CdoB protein catalyzed the oxygenation of cysteine to CSA, thereby performing the predicted reaction (Fig. 2A). The dependency of the enzyme activity on the concentration of supplied substrate showed that cysteine concentrations above 750 μM led to substrate saturation and a constant velocity of the enzymatic reaction (Fig. 2A). The maximal specific activity of CdoB for cysteine as the substrate was determined to be 0.73 μmol CSA/mg protein/min; the K_m, 2.85 mM; the k_{cat}, 0.25 s^{-1}; and the catalytic efficiency, k_{cat}/K_m, 0.087 s^{-1} mM^{-1}. Further analysis of the enzyme revealed that 3MP and cysteamine were not used as substrates (data not shown).

In addition, complete inhibition of the cysteine dioxygenase reaction by addition of 50 μM 3MP was observed for the enzyme. Cysteamine and 2-mercaptoethanol also inhibited the activity, but to a lesser extent (Fig. 2B).

An iron content of 0.42 $\pm$ 0.028 mol Fe(II) per mol CdoB was determined by the method of Pierce et al. (9). Also, Fe(III) could be detected in the purified His$_6$-tagged CdoB at 0.048 $\pm$ 0.024 mol Fe(III) per mol enzyme.

To obtain information about the metal dependency of CdoB, activity assays were conducted by using different metal-specific inhibitors and by the addition of different metal ions to the purified enzyme as cofactors (Table 2 and Fig. 2B and C).

Addition of ethyl xanthate, which specifically reduces Cu(II) to Cu(I), gave a 55% lower activity. CdoB was highly susceptible to EDTA, and the addition of 50 μM EDTA resulted in a complete loss of activity. Figure 2C illustrates the effects of different metal ions on the activity of CdoB and demonstrates that the activity is enhanced not only by $FeCl_2$, but also by $CuCl_2$, $ZnCl_2$, and $NiCl_2$. These analyses also revealed that metal ion concentrations above

TABLE 2 Effect of metal-acting compounds on enzyme activities of CdoA and CdoB[a]

Effector	Relative activity (%)	
	CdoA	CdoB
Sodium ethylxanthate		
2 µM	110 ± 18.0	55 ± 1.6
4 µM	89 ± 10.8	56 ± 7.0
EDTA		
50 µM	80 ± 5.5	0
100 µM	61 ± 4.6	0
200 µM	35 ± 3.6	0

[a] Three to 5 µg purified CdoB was incubated for 30 min in MES buffer (62 mM, pH 6.5) containing 0.5 mM cysteine and 200 µM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$. CdoA activity was obtained by 30 min of incubation of 14 µg purified CdoA in MES buffer (62 mM, pH 6.5) containing 0.5 mM 3MP and 200 µM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$. Each assay was also performed without effectors, and the activity measured under these conditions was taken as 100%. The results shown represent the averages of three independent biological replicates.

200 µM inhibited the activity of the enzyme. Using three different buffer systems, the optimal pH for CdoB enzyme activity was estimated to be pH 6.8, and the highest activities were obtained in MOPS (morpholinepropanesulfonic acid) or MES buffer (data not shown).

Because CdoB possesses a cysteine residue in cupin motif 1, we analyzed whether the protein also possesses a cross-linked cofactor. For this, 5 µg of the purified protein was denatured under nonreducing conditions and subjected to SDS-13% (wt/vol) polyacrylamide gel electrophoresis. The previously described double-band migration pattern of proteins containing a Cys-Tyr cross-link (14) was not observed for CdoB under the applied conditions (data not shown).

To further characterize CdoB, the reason for the blue appearance of the enzyme was investigated. For this, the absorption spectrum of the purified enzyme (c = 1.9 mg/ml) in the wavelength (λ) range of 200 to 900 nm was measured. The spectrum showed that the enzyme absorbs light between 450 and 850 nm, with the highest absorption occurring at a λ value of 610 nm (Fig. 3). After the second measurement, the absorption of the enzyme could no longer be determined, since the protein started to denature in the cuvette and the solution became turbid. Calculating an extinction coefficient (ε) for the enzyme at a λ value of 610 nm based on the first two measurements gave a value of 3.2 mM^{-1} cm^{-1}.

Characterization of CdoA. Analysis of purified CdoA by SEC showed an experimental molecular mass of 46.8 ± 0 kDa (see Fig. S5 in the supplemental material). Since the calculated molecular mass of a hexahistidine-tagged CdoA monomer is 23.5 kDa, the experimental molecular mass showed that CdoA apparently has a dimeric quaternary structure *in vitro*.

The capability of CdoA to oxidize 3MP to 3SP was previously revealed by an *in vivo* enzyme assay cultivating cells of *E. coli* Top10(pBBR1MCS-3::*cdoA*) in M9 medium containing 3MP (17). This unusual catalysis was confirmed in the study by an *in vitro* enzyme assay using the purified protein. In addition to 3MP, cysteine was also used as a substrate by CdoA, whereas no hypotaurine could be detected when cysteamine was supplied as the substrate. For both substrates, the substrate saturation followed hyperbolic curves. The highest activities were measured at concentrations of 0.75 mM for 3MP and 1 mM for cysteine, whereas higher concentrations led to substrate saturation and a constant velocity of

the enzymatic reaction (Fig. 4A and B). The maximal specific activity of CdoA for cysteine as the substrate was determined to be 0.05 µmol CSA/mg protein/min; the K_m, 6.7 mM; the k_{cat}, 0.01 s^{-1}; and the catalytic efficiency, k_{cat}/K_m, 0.002 s^{-1} mM^{-1}. When 3MP was used as a substrate, the enzyme showed a maximal specific activity of 2.2 µmol 3SP/mg protein/min, a K_m of 5.7 mM, a k_{cat} of 0.82 s^{-1}, and a catalytic efficiency, k_{cat}/K_m, of 0.144 s^{-1} mM^{-1}.

The inhibitory effects of various concentrations of different thiols on CdoA activity using cysteine as the substrate were tested. The results are illustrated in Fig. 4C and demonstrate that addition of 0.5 mM cysteamine, 3MP, or 2-mercaptoethanol led to a significant decrease of enzyme activities. The inhibitory effect of cysteine on enzymatic formation of 3SP was also tested. In comparison to the other thiols, the addition of cysteine resulted in a minor decrease of activity.

The optimal pH for activity was determined using a bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane/tris(hydroxymethyl)methane (Bis-Tris/Tris) broad-range buffer system (pH 5.5 to 9.0), and the highest activity occurred at pH 6.5.

An iron content of 0.38 ± 0.04 mol Fe(II) per mol CdoA was revealed by the method of Pierce et al. (9). No Fe(III) could be detected in CdoA.

The dependency of CdoA activity on divalent transition metals was demonstrated by the inhibitory effect of the chelating compound EDTA, which resulted in a 40% decrease of activity at a concentration of 100 µM. In contrast, sodium ethyl xanthate did not exhibit any inhibitory effect (Table 2).

To make a reliable statement about the importance of the iron cofactor for activity of CdoA, the metal center had to be completely removed. Thereafter, the apoenzyme was complemented with different metal ions to prove the reconstruction of a functional enzyme. For removal of the iron cofactor, a dialysis approach against 1,10-phenanthroline was used. After 30 min, it could be observed that the H_2O control stayed colorless, while the 62.5 µM standard solution of $FeCl_2$, as well as the enzyme solution (c = 2 mg/ml), became red (see Fig. S6 in the supplemental material). This red coloration disappeared after 2 to 3 h. At this time, the dialysis buffer was exchanged for pure 50 mM Tris-HCl buffer (pH 7.5), in which the solution containing dialysis tubes was dialyzed overnight. Before the dialyzed enzyme solution was used for further experiments, the enzyme was concentrated and transferred to 62 mM MES buffer (pH 6.5). After dialysis, the enzyme showed an Fe(II) content of 0.11 ± 0.013 mol Fe(II) per mol enzyme. This is equal to a decrease of the iron content of >70%.

To prove the dependency of the CdoA activity on the iron cofactor, the dialyzed enzyme was applied in activity assays to which different divalent metal ions Cu(II), Fe(II), Mg(II), Mn(II), Ni(II), and Zn (II) were added in a final concentration of 0.05 to 1.5 mM (Fig. 5). As a reference value, the activity of the dialyzed enzyme was determined without the addition of any metal ion. The dialyzed CdoA could reach an activity of only 0.29 ± 0.09 µmol 3SP · mg^{-1} · min^{-1}, which corresponds to 19.5% of the activity of the nondialyzed enzyme when no divalent metal ion was added (Fig. 5A). By addition of exogenous ferrous iron, the activity of the dialyzed enzyme could be restored, reaching the highest activity of 1.74 ± 0.15 µmol 3SP · mg^{-1} · min^{-1} when 400 µM Fe(II) was added. This corresponds to an activity 5.87-fold higher than the activity without addition of any divalent metal ion. In the case of the nondialyzed enzyme, the highest activity of 1.88 ± 0.14 µmol 3SP · mg^{-1} · min^{-1} was achieved by addition of
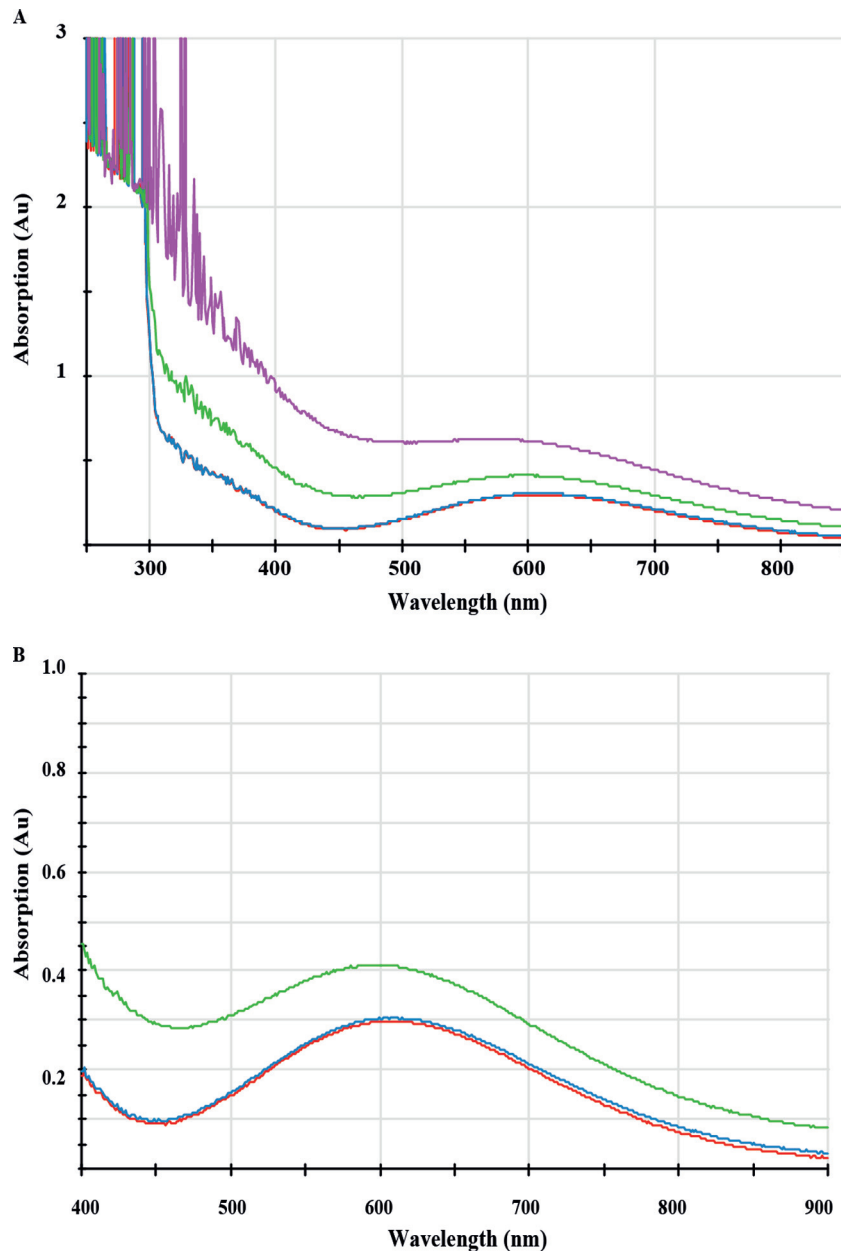
FIG 3 Absorption spectrum of purified His$_6$-tagged CdoB ($c = 1.9$ mg/ml) measured in the wavelength range of 200 to 900 nm in an Evolution 100 UV-Vis spectrophotometer in the scan mode at a constant temperature of 30°C. (A) Wavelength range of 200 to 850 nm. (B) Wavelength range of 400 to 900 nm. The same sample was measured four times in the spectrometer (red, first measurement; blue, second measurement; green, third measurement; purple, fourth measurement). Au, arbitrary units.

200 µM Fe(II). Concentrations higher than 400 µM Fe(II) led to a decrease of enzymatic activity (Fig. 5A). Only copper was able to partly restore enzymatic activity, reaching a value of 1.09 ± 0.19 µmol 3SP · mg^{-1} · min^{-1} when 1.5 mM Cu(II) was added (Fig. 5B). Mg(II), Mn(II), Ni(II), and Zn(II) failed to restore enzymatic activity (Fig. 5C to F).

DISCUSSION

Cysteine dioxygenases are nonheme Fe(II)-dependent enzymes catalyzing the sulfoxidation of cysteine to CSA. The enzyme is well known in mammals, and details of the structures have been re-ported for Cdos from rat, mouse, and human (15). Bacterial Cdos were discovered only recently, with detailed analyses reported for the enzymes from the Gram-positive bacteria *Bacillus cereus*, *Bacillus subtilis*, and *S. coelicolor* (11). The investigated bacterial enzymes showed high specificity for cysteine, and the kinetic properties were very similar to those of the Cdo from rat, indicating that these proteins were indeed bona fide Cdos (11). During our studies on the microbial catabolism of TDP and DTDP, we identified genes coding for Cdo homologues in *V. paradoxus* TBEA6 (17, 52), *R. eutropha* H16 (17), and *A. mimigardefordensis* DPN7^T (18, 51). However, the putative Cdos from *A. mimigardefordensis*
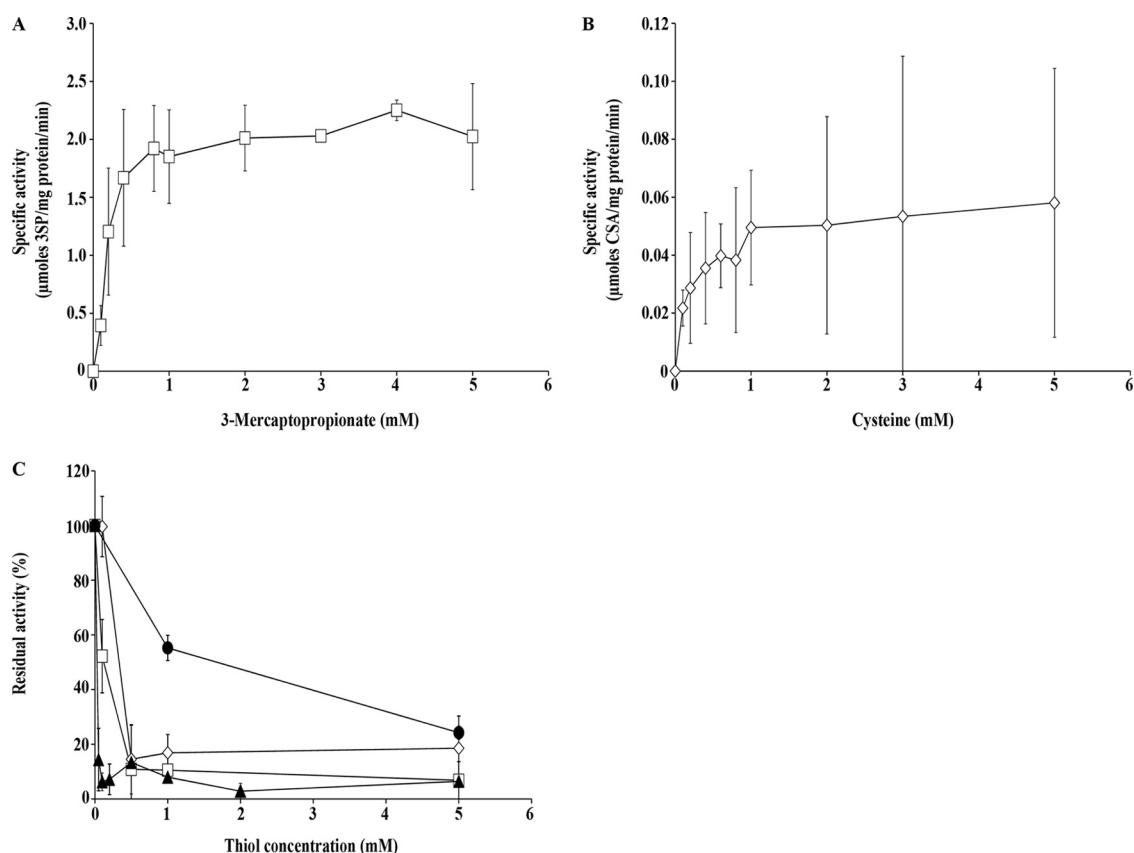
FIG 4 Enzyme activity assay of recombinant CdoA. (A and B) Dependency of CdoA enzyme activity on various concentrations of the substrates 3MP (A) and cysteine (B). Ten micrograms of purified CdoA was incubated in MES buffer (62 mM, pH 6.5) containing various concentrations of the substrate (0.05 to 5 mM), 12.5 μM bathocuproine disulfonic acid disodium salt, and 400 μM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ and shaken for 30 min at 500 rpm in a thermoblock (model MHR10; HLC BioTech, Bovenden, Germany). (C) The inhibitory effects of various concentrations of cysteine (●), 3MP (□), cysteamine (◇), and 2-mercaptoethanol (▲) on CdoA enzyme activity were also measured. For this, 3 μg of the purified protein was incubated for 30 min in MES buffer (62 mM, pH 6.5) containing 0.5 mM cysteine, 12.5 μM bathocuproine disulfonic acid disodium salt, and 200 μM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$. The inhibitory effect of cysteine was estimated using 3MP as the substrate (0.5 mM). CdoA activity measured in the absence of any effector was taken as 100%. The results shown represent the averages ± SD of three independent biological replicates.

DPN7[T] and *V. paradoxus* TBEA6 only catalyzed the sulfoxidation of 3MP to 3SP and were therefore referred to as 3MP dioxygenases (Mdo). A special case is the Cdo homologue from *P. aeruginosa* (19). This enzyme has been reported to convert 3MP, as well as cysteine, with the catalytic efficiency for 3MP being 37-fold higher than that for cysteine; therefore, it is also referred to as an Mdo.

In silico analysis of the *R. eutropha* H16 genome sequence revealed two genes encoding Cdo-homologous enzymes. Alignment of the amino acid sequences revealed significant differences, particularly in the cupin motifs, which, especially in the eukaryotic Cdos, are highly conserved (see Fig. S2 in the supplemental material). In addition, CdoA and CdoB varied not only in their primary structures (see Fig. S1 in the supplemental material), but also in the localization of the genes and in the sizes and the molecular masses of the translational products. Characteristic differences between CdoA and CdoB are summarized in Table 2.

The heterologous expression of both genes in *E. coli* and the enzymatic characterization of the purified hexahistidine-tagged fusion proteins revealed further differences between the two homologues of *R. eutropha* H16. CdoA catalyzed the sulfoxidation of cysteine and 3MP, where the catalytic efficiency (k_{cat}/K_m) of CdoA was 72-fold higher with 3MP than with cysteine. In contrast, CdoB

converted only cysteine, but with a catalytic efficiency 45 times higher than that for CdoA with this substrate. The physiological role of CdoA, similar to that of the Mdo of *P. aeruginosa*, is not clear, since 3MP does not occur in the normal metabolism of the bacterium and both are unable to use 3MP as a sole carbon source (19, 38). Nevertheless, investigation of CdoA and CdoB might be very useful for the production of polythioesters (PTEs) in *R. eutropha* H16. Since 3MP can function as a precursor for PTE synthesis, deletion of *cdoA* could lead to increased synthesis of PTEs, while the conversion of cysteine to CSA can still be performed by CdoB.

The kinetic properties of CdoA and CdoB were very similar to those of the previously investigated bacterial Cdos (11), especially the values for CdoA kinetics, which were almost identical to those of the recently described Mdo from *P. aeruginosa* (19).

The catalytic activity of CdoA with 3MP is very unusual, because the generally proposed reaction mechanisms (1, 6, 7, 10) assumed that the cysteine amino group is coordinated with the metal cofactor. Furthermore, the absolute requirement for the amino group for catalytic competence of mouse Cdo was previously shown by Pierce et al. (9), using electron paramagnetic resonance (EPR) analysis. An alternative chelating mode, where sul-
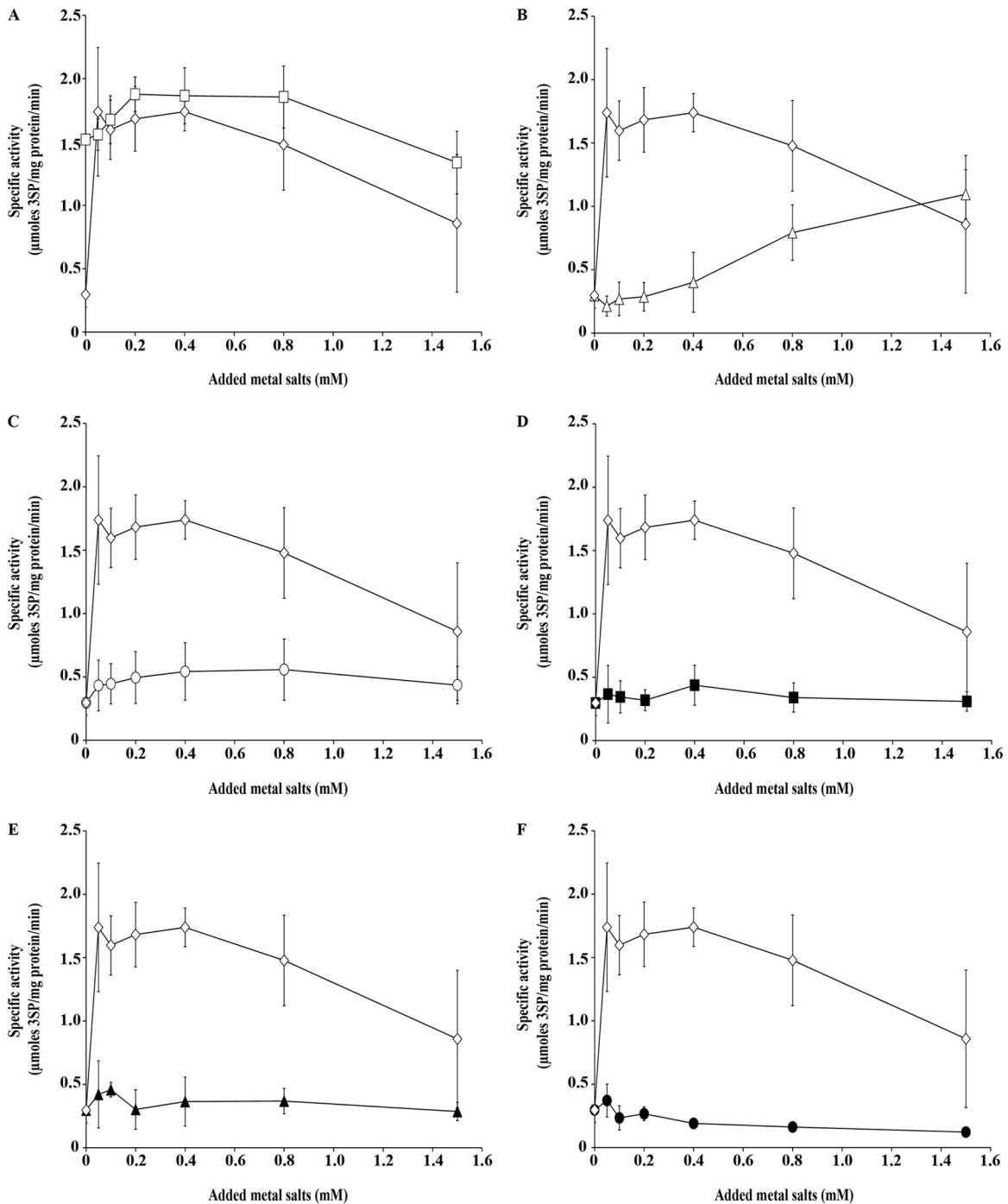
FIG 5 Complementation experiments with recombinant CdoA and recombinant dialyzed CdoA with 3MP as the substrate and addition of Fe(II) before (□) and after (◇) dialysis of the enzyme (A), Cu(II) (△) compared to Fe(II) (◇) added to the dialyzed enzyme (B), Mg(II) (○) compared to Fe(II) (◇) added to the dialyzed enzyme (C), Mn(II) (■) compared to Fe(II) (◇) added to the dialyzed enzyme (D), Ni(II) (▲) compared to Fe(II) (◇) added to the dialyzed enzyme (E), and Zn(II) (●) compared to Fe(II) (◇) added to the dialyzed enzyme (F). Ten micrograms of purified CdoA was incubated in MES buffer (62 mM, pH 6.5) containing 1 mM 3MP and various concentrations (0 to 1.5 mM) of the respective metal salt ($CuCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$) and shaken for 30 min at 500 rpm in a thermoblock (model MHR10; HLC BioTech, Bovenden, Germany). The results shown represent the averages ± SD of three independent biological replicates.

fur and a carboxylate oxygen bind to the metal cofactor, was considered by McCoy et al. (6).

Analysis of the degrees of polymerization of both enzymes by SEC showed that CdoA is a dimer and CdoB a trimer *in vitro*. So

far, most Cdo homologues have been detected as monomers (6, 7). One exception is the investigation by Ye et al. (7), who analyzed the human Cdo homologue and mutations thereof. They also observed a dimeric structure for one of the mutated enzymes, which

showed the same catalytic efficiency and metal incorporation characteristics as the monomeric enzymes. Since these described enzymes are all of eukaryotic origin, a direct comparison is not convenient. The previously described prokaryotic proteins have not been examined for their degrees of polymerization (11, 15, 17). The degree of polymerization could also be influenced by the recombinant host in which the enzyme is expressed.

Determination of the iron contents in CdoA and CdoB showed a submolar amount of Fe(II) present in the enzymes. This was also described for various other Cdo orthologues, e.g., from *Rattus norvegicus* (12, 56), *Mus musculus* (6), and human (7). Generally one would assume a ratio of 1 mol Fe(II)/mol enzyme if all enzyme molecules are present in their active forms. A reason for the submolar amount of iron in the enzyme might be too little iron in the culture medium, loss of iron during purification, or occupation of the active centers of the remaining Cdo molecules by other metals.

All previously described Cdos were characterized as strictly dependent on ferrous iron, and it has often been reported that IMAC-purified Cdos showed no catalytic activity until exogenous ferrous iron was added, whereas the addition of other transition metals failed to restore the activity (10–12). The experiments conducted in this study showed that the enzymes CdoA and CdoB are active after purification by IMAC, also without addition of exogenous divalent metal ions. Nevertheless, it could be verified that the enzyme activity of CdoB significantly increased after addition of $FeCl_2$, $CuCl_2$, $NiCl_2$, or $ZnCl_2$.

The inhibition studies with EDTA showed a complete loss of activity of CdoB even at low concentrations of EDTA (50 µM). In contrast, CdoA still retained 80% of its original activity at this EDTA concentration. This indicates stronger binding of metal ions to CdoA than to CdoB. Since 1,10-phenanthroline is a stronger chelating agent than EDTA, an approach with 1,10-phenanthroline was developed. The almost complete loss of activity of CdoA after removal of the iron by dialysis against buffer containing 1,10-phenanthroline demonstrated that iron is indeed present in the catalytic center of the enzyme. By addition of exogenous ferrous iron with an optimal concentration of 200 to 400 µM, full restoration of activity of the dialyzed enzyme was achieved. When the dialyzed enzyme was complemented with other divalent metal ions, the activity was only marginally enhanced by addition of Cu(II). A reason for this observation might be that Cu(II) is the only metal ion besides Fe(II) that is able to have 6 neighboring atoms and thereby build stable coordinated complexes with the His residues of the protein, the substrate, and O_2. Another explanation could be an undesired interaction of the buffer component MES with the tested cations, which was described by Good et al. in 1966 (57).

CdoB showed blue color after purification by IMAC. Because blue proteins are in general associated with Cu(II) content, as in plastocyanin (58), this observation is unusual for an enzyme that is predicted to be iron dependent, like CdoB. Nevertheless, there are some examples of other blue iron proteins in the literature, e.g., the 4-hydroxyphenylpyruvate dioxygenase of *Pseudomonas* sp. strain P.J. 874. The color corresponded to an absorption maximum at 595 nm and was ascribed to tyrosyl-Fe(III) charge transfer bands (59, 60). The blue color of CdoB most likely also results from a charge transfer complex between Fe(III) and a potent amino acid residue. CdoB showed an absorption maximum at 610 nm. This is very similar to the absorption maximum observed for the 4-hydroxyphenylpyruvate dioxygenase. The first report of such a blue Cdo was published by Gardner et al. (61). The aerobic addition of cysteine to an as-isolated mouse Cdo was found to change the color of the purified protein solution from pale yellow to blue. Because the electronic absorption and the magnetic-circular-dichroism spectroscopic signatures were very similar to those of a well-characterized Fe(III) superoxide reductase, the authors proposed that the visible color was caused by a cysteinyl-Fe(III) charge transfer. However, further analyses of CdoB must be performed to reveal which amino acid residue is involved in this charge transfer complex.

ACKNOWLEDGMENTS

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