Evidence for Cysteine Persulfide as Reaction Product of L-Cyst(e)ine C-S-Lyase (C-DES) from Synechocystis

ANALYSES USING CYSTINE ANALOGUES AND RECOMBINANT C-DES

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The pyridoxal phosphate-dependent monomeric L-cyst(e)ine/cysteine (C-DES), previously isolated from Synechocystis PCC 6714 by its capacity to direct [2Fe-2S] cluster assembly of ferredoxin in vitro (Leibrecht, I., and Kessler, D. (1997) J. Biol. Chem. 272, 10442-10447), has now been cloned, sequenced, and overexpressed in Escherichia coli. The amino acid sequence of C-DES was found to be nearly identical (92% identity) to the open reading frame srl2143 of Synechocystis PCC 6803 and showed a more distant relationship to the NifS family of proteins (about 27% identity). Recombinant C-DES displayed activities equal to the isolate from Synechocystis in terms of the cyst(e)ine lyase reaction and holoferrredoxin formation which recommended its use for functional and mechanistic studies. Investigation of the substrate spectrum for β-elimination found L-cysteine to be a poor substrate (kcat ≈ 0.15 s⁻¹) in contrast to L-cystine (kcat ≈ 36 s⁻¹) and several related compounds. Of these compounds, desamino-cysteine ([S-carboxyethylthio]-L-cysteine) was used for C-DES-mediated persulfide generation. Stabilization of the linear persulfide 3-(disulfanyl)-propionic acid was achieved by cyclization as a novel intramolecular trapping reaction; this yielded 1,2-dithiolan-3-one which was isolated and identified by chemical analyses.

Continuing interest in structure and function of iron-sulfur proteins (1) has recently been extended to the biosynthetic problem of how the Fe-S cluster moieties are introduced into the proteins. Genetic studies on the biosynthesis of the nitrogenase apo-iron protein have investigated the specificity of C-DES with respect to the preferred L-cystine to L-cysteine (7). C-DES showed a more distant relationship to the NifS family of proteins (about 27% identity). Recombinant C-DES displayed activities equal to the isolate from Synechocystis in terms of the cyst(e)ine lyase reaction and holoferrredoxin formation which recommended its use for functional and mechanistic studies. Investigation of the substrate spectrum for β-elimination found L-cysteine to be a poor substrate (kcat ≈ 0.15 s⁻¹) in contrast to L-cystine (kcat ≈ 36 s⁻¹) and several related compounds. Of these compounds, desamino-cysteine ([S-carboxyethylthio]-L-cysteine) was used for C-DES-mediated persulfide generation. Stabilization of the linear persulfide 3-(disulfanyl)-propionic acid was achieved by cyclization as a novel intramolecular trapping reaction; this yielded 1,2-dithiolan-3-one which was isolated and identified by chemical analyses.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/The EBI Data Bank with accession number(s) AF061964.

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The abbreviations used are: C-DES, L-cyst(e)ine C-S-lyase; HPLC, high performance liquid chromatography; MS, mass spectrometry; GC, gas chromatography; PAGE, polyacrylamide gel electrophoresis; PLP, pyridoxal phosphate; Mops, 3-(N-morpholino)propanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; ATP/oS, adenosine 5′-(α-thio)triphosphate; kb, kilobase pair(s).

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Printed in U.S.A.
L-Cystine was from Serva, L-cysteine-HCl H2O from Fluka, and S-
ethylcysteine, S-methylcysteine, L-djenkolic acid, and L-cystathionine were from Sigma. Other (bio)chemicals and enzymes were from Boehringer-Mannheim, New England Biolabs, Sigma, or Aldrich.

Peptide Sequencing—Various peptides were HPLC purified from a trypsin digest of 200 mg of C-DES from a 190 g Synechocystis PCC 6714 culture subjected to Edman degradation by R. Frank (Zentrum für Molekulare Biologie, Heidelberg). A gas-phase sequenator and on-line identification of the phenylthiohydantoin by HPLC was employed. NH2-terminal sequencing of C-DES expressed in E. coli employed the chromatofo-
focused fractions (see below).

Diagnosis of Expression Plasmid pSA16—In general, standard procedures as described by Sambrook et al. (10) were used.

Starting with 50 μg of total Synechoystis DNA the 7.6-kbp size fraction of a HindIII digest was ligated into pUC19. After transformation of XL1Blue MRF

Sph sites of pUC19) to generate two subclone series by exonuclease III

filling in the termini followed by blunt-end ligation into pUC19 cut with

Hin

m

I and

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To clone

Cloning and DNA Sequencing of the C-DES Gene—To clone

the C-DES gene from

Synechocystis PCC 6714 we attempted to

gain some sequence data for the purified protein. NH2-terminal

sequencing failed, suggesting a blocked terminus, but some

information could be obtained from tryptic peptides (see Fig. 2).

Based on the peptide sequence Glu-Val-Asp-Tyr-Tyr-Ala- a 64-

fold degenerate pool of oligonucleotides (17-mers) was synthe-

sized. This probe was labeled with digoxigenin and used to

analyze Southern transfers of restricted total Synechocystis

DNA. One signal for each digest was detected and the 7.6-kbp

HindIII fragment was selected for the cloning experiments. After ligation into the HindIII site of pUC19 and transformation

into E. coli XL1Blue MRF+, about 3000 colonies were

screened to obtain two positive clones. Southern analysis of the

restricted plasmids showed that they contained identical

HindIII fragments. The hybridization site of the oligonucleotide

probe was mapped to a 1.1-kbp

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RI subfragment internal to

a 4.5-kbp HindIII-BamHI fragment (Fig. 1). This 4.5-kbp segment

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A search through the data bases employing a FASTA routine

(15) revealed only limited homologies except for the corre-

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(d) because of an extra residue inserted in the PLP binding motif and lack of a cysteinyl residue located in the equivalent position to the covalent catalytic residue (Fig. 2).

Heterologous Overexpression of C-DES in E. coli—To express

C-DES in E. coli, the 1.6-kbp SspI-Aval fragment was cloned downstream of the lac promoter of pUC19 in the appropriate orientation (field plasmid pSA16) (Fig. 1). Plasmid pSA16 was found to make up about 5% of the extract proteins obtained from pSA16 transformed E. coli PR745 cells. Its cysteine lyase activity (liberating pyruvate) was conveniently measured by a coupled optical assay using the lactate dehydrogenase reaction as indicator. Starting with 10 g of wet cells about 22 mg of pure C-DES were obtained which displayed UV-VIS absorption characteristics consistent with the expected content of PLP ($A_{421}$% = 1.4; Ref. 17). However, the preparation consisted of

FIG. 1. Restriction map of cloned Synechocystis PCC 6714 DNA

harboring the C-DES structural gene and construction of expres-

sion plasmid pSA16. Only relevant restriction sites are shown. Sequenced DNA segments covered by exonuclease III-generated subclones are indicated by the two bars above the shown HindIII-BamHI fragment. Structural genes and direction of transcription are indicated by arrows. The vector portion of pSA16 is shown as thinner line and includes the ampicillin (Ap) resistance gene.

Results

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FIG. 2. Deduced amino acid sequence of C-DES and sequence

alignments with the slr2143 gene product of Synechocystis PCC

6803 (accession number D90908), isopenicillin N-epimerase of

Streptomyces clavuligerus (CefD, accession number M32324), and

one of the E. coli NiFS homologues (NiFS, accession number

D90811). Known peptide segments of the C-DES sequence are

underlined where the amino acid identity could not be

unambiguously identified. NH2 termini found with C-DES overexpressed in E. coli were either as deduced (but with the initiator methionine lacking; minor species) or Met*-Asn* (major species, see text). Sequence similarities were searched with the FASTA program (15) and aligned with the CLUSTAL program (29). The PLP binding motif is marked with asterisks. Gaps are shown with dashes. Shaded residues are conserved among at least 3 of the sequences displayed. For isopeni-

cillin N-epimerases and NiFS proteins the representative with the best score is displayed although no biochemical data are available for the NiFS homologue cited. The cysteinyl residue corresponding to the covalent catalytic residue for this NiFS homologue (5) is indicated by an arrow.

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two closely related species which could be partially separated by chromatofocusing.

The major species (higher pI, about 80%) showed the NH₂-terminal sequence Met-Asn-Leu-Ile-Pro-, which implies usage of the GTG codon of Val-5 in the open reading frame as start codon. Preceded by 5'-CT and followed by A-3', this GTG codon fits the known preference for initiation in *Synechocystis* at sites with the consensus 5'-YY( initiation codon)R-3' (18). This site is most probably also used in *Synechocystis* cells. The minor protein species (about 20%) showed the NH₂-terminal sequence Ala-Asp-Pro-Val- which implies usage of the ATG codon of Met1 in the open reading frame as start codon; the mature protein lacks the initiator methionine.

Using a chromatofocused preparation of the major species the A₂₈₀ ratio of C-DES without or with 6 M guanidinium chloride was found to be 1.05. Based on a molecular mass of 42,772 Da and a calculated A₂₈₀(1%) of 13.8 for a 6 M guanidinium chloride solution (19) this gives an A₂₈₀(1%) value of 14.5 for the native C-DES (major species).

Both enzyme species were found to be equally active whether assayed for cystine lyase in the coupled optical assay (7 units/mg) or holoferredoxin formation (8 mg of holoferredoxin h⁻¹ mg⁻¹). These data are congruent with the specific activity determined for the *Synechocystis* isolate (7).

β-Elimination Reaction with L-Cystine and Related Compounds—To investigate the substrate requirements of C-DES, several compounds related to L-cystine were examined for pyruvate formation in the coupled optical lyase assay (Table I).

Besides L-cystine, L-djenkolic acid (which also harbors two L-cysteinyl-moieties) finally yielded 2 mol of pyruvate per mol of substrate. This stoichiometry indicates further enzymatic (and possibly also spontaneous) reactions of the primary elimination products. With our attention directed to the initial reaction of C-DES with L-cystine, asymmetric derivatives thereof were considered as useful. Desaminocystine, decarboxycystine, and meso-cystine were all synthesized by reaction of the appropriate thiol with S-(2,4-dinitrophenylthio)-L-cysteine and were found to be readily accepted as substrates yielding 1 equivalent of pyruvate (Table I).

Besides djenkolic acid, further non-disulfidic compounds structurally related to L-cystine were examined (Table I). This revealed that C-DES activity is confined to compounds with at least one L-cysteinyl moiety and various S-substituents but not necessarily disulfides. However, L-cysteine was by far the least efficient of the compounds tested; with L-cystathionine and S-(methylcysteine) moderate efficiency was observed which argues against identity of C-DES with cystathionase or S-alkylcysteine-lyase.

**Identification of a Persulfide Product from Reaction of C-DES with Desaminocystine**—Toward identification of a persulfide product desaminocystine with its favorable kinetic properties (Table I) was selected among the substrates tested. The stoichiometric yield of 1 equivalent of pyruvate suggested formation of 3-(disulfanyl)-propionic acid which was supposed to cyclize spontaneously affording 1,2-dithiolan-3-one (1; see Scheme I and Fig. 3) as a stable derivative of the linear

| Substrate | Stoichiometric yield | Kinetic parameters |
|-----------|---------------------|--------------------|
|           | mod pyruvate/mol substrate | kₘ₀ | kₗ₀ | kₗ₀/kₘ₀ |
| L-Cystine | L-Cy-S | 2.0 | 0.5 | 29 | 58 |
| D-Cystine | D-Cy-S | 0 | 0 | 0 |
| Meso-cystine | L-Cy-S | 1.0 | 2.2 | 29 | 13 |
| L-Djenkolic acid | L-Cy-S | 2.0 | 0.7 | 50 | 71 |
| Desaminocystine | L-Cy-S | 1.0 | 0.5 | 29 | 58 |
| Decarboxycystine | L-Cy-S | 1.1 | 0.8 | 14 | 18 |
| L-Cystathionine | L-Cy-S | 1.0 | 1.2 | 7 | 6 |
| S-Ethyl-L-cysteine | L-Cy-S | 1.1 | 7.3 | 37 | 5 |
| S-Methyl-L-cysteine | L-Cy-S | 1.2 | 5.7 | 20 | 3.5 |
| L-Cysteine | L-Cy-SH | 1.0 | — | 0.15 |

* L-Cy-S/D-Cy-S- denote L-cysteinyl and D-cysteinyl moieties.
* Below detection limit of 0.01 s⁻¹.
* Concentration dependence on L-cysteine as substrate did not display Michaelis-Menten characteristics.
* Measured with 2 mM cysteine.

| Substrate Structure | Stoichiometric yield | kₘ₀ | kₗ₀ | kₗ₀/kₘ₀ |
|---------------------|---------------------|-----|-----|---------|
| L-Cystine | L-Cy-S | 1.0 | 1.0 | 36 | 36 |
| D-Cystine | D-Cy-S | 0 | 0 | 0 |
| Meso-cystine | L-Cy-S | 1.0 | 2.2 | 29 | 13 |
| L-Djenkolic acid | L-Cy-S | 2.0 | 0.7 | 50 | 71 |
| Desaminocystine | L-Cy-S | 1.0 | 0.5 | 29 | 58 |
| Decarboxycystine | L-Cy-S | 1.1 | 0.8 | 14 | 18 |
| L-Cystathionine | L-Cy-S | 1.0 | 1.2 | 7 | 6 |
| S-Ethyl-L-cysteine | L-Cy-S | 1.1 | 7.3 | 37 | 5 |
| S-Methyl-L-cysteine | L-Cy-S | 1.2 | 5.7 | 20 | 3.5 |
| L-Cysteine | L-Cy-SH | 1.0 | — | 0.15 |

**Table I**

Substrate properties of L-cystine and related compounds

Data refer to the total amount or the initial rate of pyruvate formation observed with the lactate dehydrogenase indicator reaction in the coupled optical lyase assay of C-DES (see "Experimental Procedures").
Generation of a Substrate-derived Persulfide by C-DES

persulfide.

Since only small amounts of products were available from enzymatic reactions their identification was deduced by chromatographic analyses referring to chemically synthesized reference compounds. Synthesis of 3-(disulfanyl)-propionic acid from mercaptopropionic acid, as outlined in Scheme I, furnished a crude CS₂-insoluble product which was fractionated by C18 reversed-phase HPLC and found to contain principally three components depicted in Scheme I: the dithiolanone 1, the tetrathiobis propionic acid 2, and the trithiobis propionic acid 3. Formation of byproducts 2 and 3 can be explained by bimolecular reactions of the persulfide molecules (see Scheme I). These latter reactions should be largely suppressed by low temperature and dilution of the reactants in the enzymatic reactions.

The enzymatic conversion of desaminocystine was carried out at 5 °C and pH 6. This pH value was chosen to suppress disproportionation of the asymmetric disulfide substrate and to favor the cyclization reaction of the presumed linear persulfide product; it compromised between the stabilizing effects mentioned and the lyase activity of C-DES which is optimal at pH 8 and decreases to 6% at pH 6.0 or 9.6. The reactions were stopped by additions of HCl and ether. After thorough mixing, the phases were separated and their content analyzed (see Fig. 3). Remarkably, the sulfur-containing products (which were routinely detected and quantified by HPLC analysis) were found to be identical with chemically synthesized compounds 1 to 3 (Scheme I and Fig. 3) despite the entirely different reaction conditions. To verify the identity of the enzymatically formed key compound 1 with 1,2-dithiolan-3-one a HPLC-purified sample of 105 nmol was analyzed by GC-MS. It proved to be indistinguishable from the chemically synthesized reference compound affording the molecular ion \( m/z \) 120 and fragments \( m/z \) 64 \([S-S]^+\) and \( m/z \) 55 \([O=C-CH-CH_2]^+\) for the equivalent GC-fraction at 8.2 min of the temperature program.

An approximately constant fractional amount (see below) of dithiolanone 1 was found in five experiments with the initial concentration of desaminocystine being varied from 0.1 to 1 mM, the concentration of C-DES varied from 0.03 to 0.27 mg/ml and with reaction times from 2 to 10 min. (Conversion of desaminocystine was between 11 and 100% complete for these experiments.) However, shift of the reaction temperature to 25 °C with otherwise identical conditions lowered the fractional amount of 1 by a factor of 1.6. Control reactions without C-DES did not yield any product.

A typical experiment performed at 5 °C is presented in Figs. 3 and 4. With this reaction an amount of pyruvate corresponding to complete conversion of desaminocystine was found in the aqueous phase (Fig. 3). HPLC analysis (Fig. 4) of the ether phase revealed that 71% of the sulfur-containing products (referring to the number of moles formed) could be recovered as dithiolanone 1 (Fig. 3). The total amount of sulfur represented by products 1 to 3 slightly exceeded the amount contained initially in the substrate. This must be due to some insufficiency of our quantification protocol but nevertheless excludes the possibility that relevant amounts of further products had formed.

To investigate the stoichiometry of pyruvate formation versus formation of products 1 to 3, a kinetic experiment was performed (Table II). The ratio of dithiolanone 1 to pyruvate was maximal for the early samples (Table II); this indicates the direct formation of 1,2-dithiolan-3-one from the linear persulfide 3-(disulfanyl)-propionic acid. Therefore, a substrate-derived persulfide is generated as an obligate intermediate along the reaction pathway of C-DES.
TABLE II

| Time (min) | Products formed | Ratio 1/2 | Ratio 1/pyruvate |
|-----------|-----------------|-----------|-----------------|
|           | Pyruvate | 1 | 2 | 3 | 1 + 2 × 2 + 2 × 3 |      |
| 2         | 8      | 5.0 | 0.8 | 1.1 | 8.8 | 0.63 |
| 6         | 15     | 8.2 | 2.6 | 1.8 | 17.0 | 0.55 |
| 12        | 22     | 9.9 | 2.9 | 4.3 | 24.3 | 0.45 |
| 18        | 26     | 10.1 | 2.4 | 4.9 | 24.7 | 0.39 |
| 25        | 30     | 11.4 | 3.3 | 6.0 | 30.0 | 0.38 |

\*The amounts given refer to 0.75 ml of sample volume.
\*\*3-(Disulfanyl)-propionic acid represented by products 1 to 3 according to Scheme I. Two molecules of the linear persulfide 3-(disulfanyl)-propionic acid are required to form one molecule of 2 or 3.

DISCUSSION

Using the cystine analogue desaminocystine (S'-2-carboxymethylthio)-L-cysteine we now established that a persulfidic product, 3-(disulfanyl)-propionic acid, is stoichiometrically formed by C-DES. This linear persulfide was isolated as 1,2-dithiolan-3-one (formed by C-DES). This linear persulfide was isolated as 1,2-dithiolan-3-one (Fig. 5, 1) and was identified as a product of the persulfidic intermediate resulting in formation of a second molecule of pyruvate. With the striking preference of C-DES for cystine rather than cysteine, a role for cysteine persulfide in the overall process of Fe-S cluster formation becomes feasible which would assign cystine to the true sulfur-delivering substrate. In this context it is necessary to return to the original milieu of our holoperoxidoxin formation assay (7) with its predominantly reducing conditions. Excess glutathione was employed as thiol reagent required for apoperoxidoxin protection and guaranteed preponderance of cysteine instead of cystine. Apparent reaction of C-DES with cysteine may be made possible by the presence of catalytic amounts of cystine (Fig. 5, a; b; see also Ref. 22). The stoichiometric transfer of sulfur to the apoprotein found with the complete system should use one of the branches suggested in Fig. 5 (reactions c or b' + c'). A transient transfer of sulfane sulfur to a C-DES cysteinyl residue may additionally be involved but must be dispensable at least for the lyase reaction with non-disulfidic substrates.

It should be noted that cysteine persulfide was formerly recognized as a product of the γ-cystathionase reaction with cystine, a secondary substrate. Reactions run in the presence of iodacetate produced some S-(carboxymethylthio)-L-cysteine (22).

Through generation of cysteine persulfide C-DES shares the occurrence of a S² compound along its reaction pathway with rhodanese, mercaptopyruvate sulfur transferase, and NiFeS proteins implicated in Fe-S cluster synthesis (23, 24, 3). This may indicate that persulfidic compounds are the physiologically relevant sulfur donors. For rhodanese and NiFeS a protein persulfide is formed during catalysis which is located at a catalytically essential cysteiny1 residue (Cys-247 for bovine mitochondrial rhodanese, Ref. 25; Cys-325 for NiFeS from A. vinelandii, Ref. 4). However, the protein persulfide equilibrates with thiols contained in the medium to yield the persulfide derivatives of the thiols (6, 26) and the regenerated sulphydryl form of the enzyme. For mercaptopyruvate sulfur transferase a close relationship to rhodanese has recently been suggested from sequencing and mutagenesis studies (27).

All the experiments reported were performed using overexpressed C-DES isolated from E. coli. Recombinant C-DES proved to be indistinguishable from the original Synchocystis isolate with respect to activity. However, a minor difference was noted upon NH₂-terminal sequencing. Whereas the NH₂ terminus of C-DES isolated from Synchocystis appeared to be blocked, two species with different NH₂ termini were found for the enzyme expressed in E. coli. Presumably lacking of a typical Shine-Dalgarno sequence upstream of the C-DES gene enables the translation machinery of E. coli to use alternative start sites. Overexpression did not impair the growth rate of the host cells, thus excluding any toxic effect.

Sequence comparisons revealed that C-DES is distantly related to the NiFeS family of proteins; best matches were obtained for isopenicillin N-epimerases and NiFeS proteins in the narrow sense. Although the C-DES homologue in Synchocystis PCC 6803 has been named CefD, an isopenicillin N-epimerase function for C-DES is quite unlikely. Evidence for penicillin synthesis in cyanobacteria is lacking and a homologue for isopenicillin synthase has not been detected in the genome of Synchocystis PCC 6803 (8). The highest score for the NiFeS-like proteins contained in the data bases was obtained by comparison with a putative E. coli NiFeS protein (Fig. 2). This protein has been described as similar to the NiFeS-type cysteine sulfinate desulfinase of E. coli which yields alanine and sulfite as products (5). Interestingly, no cysteinyl residue of cysteine sulfinate desulfinase was found to be essential for activity by mutagenic replacement against alanine (5). Different members of the NiFeS sequence family evidently make use of different types of PLP chemistry.

Using recombinant C-DES it should be possible to further follow the path of sulfur from cysteine persulfide to apoperoxidoxin by analysis of sulfur-containing (protein-)intermediates. This might reveal a persulfidic derivative of apoperoxidoxin (see Fig. 5) as already discussed for apoproteins in general (6) and perhaps exemplified by the Y13C mutant of ferredoxin I of A. vinelandii (28), where the cysteiny1 persulfide might represent a dead end or side product. By mutagenesis of C-DES, residues...
essential for catalysis of the $\beta$-elimination reaction and possibly for protein-protein interaction could be identified. Along these lines we hope to gain comprehension of the perfect stoichiometry of sulfur incorporation into apoferredoxin by the catalytic action of C-DES.

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