Structural Basis for the Catalytic Mechanism of Ethylenediamine-\(N,N'\)-disuccinic Acid Lyase, a Carbon–Nitrogen Bond-Forming Enzyme with a Broad Substrate Scope

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ABSTRACT: The natural aminocarboxylic acid product ethylenediamine-\(N,N'\)-disuccinic acid [(\(S,S\))-EDDS] is able to form a stable complex with metal ions, making it an attractive biodegradable alternative for the synthetic metal chelator ethylenediaminetetraacetic acid (EDTA), which is currently used on a large scale in numerous applications. Previous studies have demonstrated that biodegradation of (\(S,S\))-EDDS may be initiated by an EDDS lyase, converting (\(S,S\))-EDDS via the intermediate N-(2-aminoethyl)aspartic acid (AEAA) into ethylenediamine and two molecules of fumarate. However, current knowledge of this enzyme is limited because of the absence of structural data. Here, we describe the identification and characterization of an EDDS lyase from Chelativorans sp. BNC1, which has a broad substrate scope, accepting various mono- and diamines for addition to fumarate. We report crystal structures of the enzyme in an unliganded state and in complex with formate, succinate, fumarate, and (\(S,S\))-EDDS. The structures reveal a tertiary and quaternary fold that is characteristic of the aspartase/fumarase superfamily and support a mechanism that involves general base-catalyzed, sequential two-step deamination of (\(S,S\))-EDDS. This work broadens our understanding of mechanistic diversity within the aspartase/fumarase superfamily and will aid in the optimization of EDDS lyase for asymmetric synthesis of valuable (metal-chelating) aminocarboxylic acids.

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succinate lyase, δ-crystallin, and 3-carboxy-cis,cis-muconate lactonizing enzyme. They share a common tertiary and quaternary fold, as well as a similar active site architecture, and process succinyl-containing substrates, leading to the formation of fumarate as the common product (except for the CMLE-catalyzed reaction, which results in the formation of a lactone).12 Current knowledge of the reaction mechanism of EDDS lyase is limited, however, because of the absence of structural data. We have therefore focused our attention on the molecular cloning of an EDDS lyase and initiated structural studies with the aim of elucidating the details of its unusual two-step addition—elimination reaction mechanism.

Here we describe the identification and cloning of the gene encoding EDDS lyase from the bacterium *Chelativorans* sp. BNC1, which was isolated from industrial sewage receiving EDTA-containing wastewater effluents.13 The enzyme has been purified to homogeneity and subjected to functional and structural characterization. It was found to accept a wide range of structurally distinct amines for addition to fumarate. In addition, we have previously determined that this *Chelativorans* enzyme also accepts a wide variety of amino acids with terminal amino groups for selective amination of fumarate, demonstrating its synthetic usefulness for the production of various important metal-chelating aminocarboxylic acids.14 Crystal structures of the enzyme in an unliganded state and in complex with formate, succinate, fumarate, AEAA, and (S,S)-EDDS were determined. These structures confirm a structural fold that is characteristic of the aspartate/fumarase superfamily and support a mechanism that involves general base-catalyzed, sequential two-step deamination of (S,S)-EDDS.

**MATERIALS AND METHODS**

**Materials.** Ingredients for buffers and media were obtained from Duchefa Biochemie (Haarlem, The Netherlands) or Merck (Darmstadt, Germany). All other chemicals used in the experiments, including the sodium salt of (S,S)-EDDS, fumaric acid, and succinic acid, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless stated otherwise. Molecular biology reagents, including restriction enzymes, polymerase chain reaction (PCR) reagents, T4 DNA ligase, and DNA and protein ladders, were obtained from Fermentas (ThermoFisher Scientific, Pittsburgh, PA) or Promega Corp. (Madison, WI). PCR purification, gel extraction, and Miniprep kits were provided by Machery-Nagel (Duren, Germany). Ni-Sepharose 6 Fast Flow and prepacked PD-10 Sephadex G-25 columns were purchased from GE Healthcare Life Sciences (Uppsala, Sweden). Primers for DNA amplification were synthesized by Eurofins MWG Operon (Cologne, Germany).

**General Methods.** Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were based on standard protocols or as suggested by the manufacturer. PCR was performed in a DNA thermal cycler obtained from Biologe (Nijmegen, The Netherlands). DNA sequencing was performed by Macrogen (Amsterdam, The Netherlands). Protein was analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions using sodium dodecyl sulfate (SDS) on precast gels containing 7.5–10% polyacrylamide (Invitrogen). The gels were stained with InstantBlue (Expedeon Inc.). Protein concentrations were determined by the Waddell method.15 Kinetic data were obtained on a V-650 spectrophotometer obtained from Jasco (IJsselstein, The Netherlands). The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus Software Ltd., Horley, U.K.) obtained from Sigma Chemical Co. Dynamic light scattering (DLS) experiments were performed using a DynaPro MS800TC instrument (Wyatt Technology Corp.) at 20 °C, and data were processed and analyzed with Dynamics software (Wyatt Technology Corp.).

**Enzymatic Synthesis of AEAA Using MAL-Q73A.** The reference compound N-(2-aminoethyl)aspartic acid [(S)-AEAA] was synthesized using the previously reported MAL-Q73A enzyme.16 A solution (80 mL) of fumarate (2 g, 17.24 mmol, 215 mM), 1,2-diaminoethane dihydrochloride (32 g, 240 mmol, 3 M), and MgCl2 (20 mM) was prepared. The pH was adjusted to 9.0 by using aqueous NaOH. The reaction was started by the addition of freshly purified MAL-Q73A enzyme (40 mg, 0.005 mol %), and the reaction mixture was incubated at room temperature. The progress of the reaction was monitored by ultraviolet–visible (UV–vis) spectroscopy. The reaction was stopped after 7 days by incubating the reaction mixture at 100 °C for 10 min, and precipitated protein was removed by filtration. An excess of amine was removed from the reaction mixture by the use of a rotary evaporator. The concentrated crude reaction mixture was dissolved in 50 mL of 1 N HCl, and the desired amino acid product was purified by cation-exchange chromatography by following a previously described protocol.16 The purified product was obtained as the bis-ammonium salt and identified as N-(2-aminoethyl)aspartic acid by 1H nuclear magnetic resonance (NMR) and 13C NMR spectroscopy and HRMS analysis. N-(2-Aminooethyl)aspartic acid: conversion 100% (7 days); 61% yield, 2.2 g; brown solid; 1H NMR (500 MHz, D2O) δ 2.66 (dd, 1H, J = 17.3, 9.0 Hz, CHCH3), 2.83 (dd, 1H, J = 17.3, 3.8 Hz, CHCH3), 3.34–3.46 (m, 4H, NHCH2CH2NH2), 3.83 (dd, 1H, J = 9.0, 3.7 Hz, CHNH3). 1H NMR signals are in agreement with the literature data.16

**Cloning of the EDDS Lyase Gene into an Expression Vector.** The amino acid sequence of the putative EDDS lyase from *Chelativorans* sp. BNC1, which was annotated as an argininosuccinate lyase, was obtained from the NCBI protein database under GenBank entry ABG61966 (NCBI reference sequence WP_011579909.1). The corresponding DNA sequence was codon-optimized for *Escherichia coli* and synthesized by Eurofins MWG Operon (Ebersberg, Germany). The gene was delivered in the pBSII SK+ vector with restriction sites for NdeI and HindIII at the 5′ and 3′ ends of the gene, respectively. The gene was amplified by PCR using primers
Ed_fv-NdeI (GGAGAAATTACATGAAACTCAACGTA-
CAGCACC) and Ed_his-st-rv-HindIII (CATAAGCTTTA-
TAAGGATAGATGATGATGCGAGATATTGCCTGCAC-
GCGCC) (the restrictions sites are depicted in bold), digested
with NdeI and HindIII, and cloned into the pBADN/Myc-His A
expression vector to obtain the pBADN(EDDS-His) construct.
The entire gene was sequenced to verify that no mutations
were introduced during the cloning procedure.

Expression and Purification of EDDS Lyase. The His6-
tagged enzyme was overproduced in E. coli TOP10 cells using
the pBADN(EDDS-His) expression plasmid. Freshly trans-
formed TOP10 cells containing this plasmid were used to
inoculate 10 mL of LB/Amp medium. After overnight growth
at 37 °C, this culture was used to inoculate 1 L of LB/Amp
medium in a 5 L Erlemeyer flask. Cultures were grown to an
A590 of 0.4–0.6 at 37 °C while being vigorously shaken, induced
with arabinose [0.04% (w/v)], and placed at 20
°C for
overnight incubation (~16 h). Cells were harvested by
centrifugation (6000g for 15 min) and stored at −20 °C until
further use.

In a typical purification experiment, cells from a 1 L culture
(~5.0 g wet weight) were thawed and suspended in 10 mL of
lysis buffer [50 mM Tris-HCl and 20 mM imidazole (pH 8.0)].
Cells were disrupted by sonication for 4 × 1 min (with a 5 min
rest between each cycle) at a 60 W output, after which
unbroken cells and debris were removed by centrifugation
(10000g for 30 min). The supernatant was filtered through a
0.45 μm-pore diameter filter and incubated with Ni-Sepharose
(1 mL slurry in a small column at 4 °C for ≥18 h), which
had previously been equilibrated with lysis buffer. The nonbound
proteins were eluted from the column by gravity flow.
The column was first washed with lysis buffer (10 mL) and then
with buffer A [50 mM Tris-HCl and 40 mM imidazole (pH 8.0),
10 mM]. Retained proteins were eluted with buffer B [50
mM Tris-HCl and 500 mM imidazole (pH 8.0), 4 mL].
Fractions were analyzed by SDS–PAGE on gels containing
10% acrylamide; those that contained purified EDDS lyase
were pooled, and the buffer was exchanged against 50 mM Tris-HCl
(pH 8.0) and 200 mM NaCl using a prepacked PD-10
Sephadex G-25 gel filtration column. The purified enzyme
yield of ~50 mg) was stored at 4 or −20 °C until further use.

Construction and Production of EDDS Lyase Mutants.
The S280A and D290A mutants of EDDS lyase were
constructed using the QuikChange site-directed mutagenesis
method (Stratagene). Plasmid pBADN(EDDS-His) was used as
a template. For the S280A mutation, the following
oligonucleotides were used as forward and reverse primers:
S′-GGCGGAACCGCCGTAGATCAGCGCCG-3′ and S′-GCGGCATGATCGACGAGTCCCGCG-3′,
respectively (the mutated codon is depicted in bold). For the D290A
mutation, the following oligonucleotides were used as forward
and reverse primers: S′-CGCAGAAGAAAAGCCGCGTAC-
CCTGGAACTAGTCGCG-3′ and S′-GGCAATCGTCTCC-
AGCTACTCCG-5′, respectively (the mutated codon is depicted in bold). DNA sequencing of
the mutant genes was performed to ensure that only the desired
mutation had been introduced.

The S280A and D290A mutants were produced and purified
using protocols similar to those used for wild-type EDDS lyase.
The S280A mutant was further purified by gel filtration chromatography with a Superdex 200 10/300 GL column (GE
Healthcare) using 0.1 M NaCl in 50 mM Tris-HCl buffer (pH
7.5) as an eluent, whereas the D290A mutant was further
purified to homogeneity by gel filtration chromatography with a
HiLoad 16/600 Superdex 200 pg column (GE Healthcare)
using 20 mM NaH2PO4 buffer (pH 8.5) as an eluent. Activity
assays were performed with freshly purified proteins (notably,
some protein precipitation was observed upon storage of the
S280A mutant, indicative of poor stability).

Enzyme Assays. Kinetic assays were performed at 25 °C in
50 mM Tris-HCl buffer (pH 8.0), revealing the increase in
absorbance at 240 nm corresponding to the formation of
fumarate (ε = 2530 M−1 cm−1). An aliquot of EDDS lyase (180
μg) was diluted into buffer (15 mL) and incubated for 30 min
at 25 °C. Subsequently, a 1 mL portion was transferred to a 10
mm quartz cuvette, and the enzyme activity was assayed by the
addition of a small quantity (0.5–20 μL) of (S,S)-EDDS from a
stock solution (10 mM). The stock solution was made up in
50 mM Tris-HCl buffer (pH 8.0). The concentrations of (S,S)-
EDDS used in the assay ranged from 0.005 to 0.2 mM.

The pH optimum of EDDS lyase was determined in 50 mM
phosphate buffers with pH values ranging from 4.4 to 9.2 at 25
°C. A sufficient quantity of enzyme was added (12 μg/mL) and
its activity assayed by adding (S,S)-EDDS from a stock solution
to a final concentration of 0.1 mM, following the increase in
absorbance at 240 nm corresponding to the formation of
fumarate. The initial reaction rates were plotted against pH.

The temperature optimum was determined in Tris-HCl
buffer (50 mM, pH 8.0), using a temperature range of 10–80
°C. At each temperature, the pH of the Tris buffer was adjusted
to the desired value of 8.0. A 1 mL portion of the buffer was
transferred to a 10 mm cuvette; a sufficient quantity of enzyme
was added (12 μg/mL), and its activity was assayed using 0.1
mM (S,S)-EDDS (2 μL of a 50 mM stock solution) as the
substrate. Substrate stock solutions were made in Tris-HCl
buffer (50 mM, pH adjusted to 8.0). The initial reaction rates
were plotted against temperature.

Enzymatic Synthesis of AEAA and (S,S)-EDDS Using
EDDS Lyase. A reaction mixture containing fumaric acid (50
mM) and ethylenediamine (10 mM) was prepared in 20 mM
NaH2PO4-NaOH buffer (pH 8.5). EDDS lyase (14 mg, 0.05
mol %) was added to start the reaction, and the reaction
volume was immediately adjusted to 50 mL with 20 mM
NaH2PO4-NaOH buffer (pH 8.5). The reaction was allowed to
proceed at room temperature. At different time points, reaction
samples (0.5 mL) were taken from the reaction mixture and
boiled for 10 min to inactivate the enzyme. The samples were
dried under vacuum and redissolved in 0.5 mL of D2O for 1H
NMR measurements.

The 1H NMR (500 MHz, deuterium oxide) signals of (S,S)-
EDDS are δ 3.57 (dd, J = 8.7, 4.3 Hz, 2H), 3.08–2.88
(4, 4H), 2.66 (dd, J = 16.2, 4.3 Hz, 2H), 2.48 (dd, J = 16.2, 8.8 Hz, 2H). The 1H NMR signals (500 MHz, deuterium oxide) of AEAA
are δ 3.43 (dd, J = 10.4, 3.7 Hz, 1H), 3.14–2.92 (3, 3H),
2.84–2.77 (m, 1H), 2.60 (dd, J = 15.5, 3.7 Hz, 1H), 2.29 (dd, J =
15.5, 10.4 Hz, 1H). After 2 h, the ratio between AEAA and (S,S)-EDDS in the reaction mixture was ~2:1, calculated by
integration of the signals at 3.43 and 3.57 ppm, respectively.

Substrate Scope and Product Identification by Liquid
Chromatography–Tandem Mass Spectrometry (LC–
MS/MS). Various amines were tested as substrates for EDDS
lyase in the addition to fumarate. Fumarate (5 mM), amine
(400 mM), and EDDS lyase (0.5 mg/mL, 0.17 mol %) in 50
mM Tris-HCl buffer (pH 8.0) were incubated at room
temperature in a 96-well plate (final volume of 150 μL).
Reactions were monitored by UV spectroscopy, following the

decrease in absorbance at 240 nm corresponding to the depletion of fumarate.

For several selected amine substrates, reactions were performed with two different substrate ratios and the products identified via LC–MS/MS. Reactions were initially performed with a 20-fold excess of amine. Fumarate (50 mM), amine (1000 mM), and EDDS lyase (0.5 mg/mL) in buffer [Tris-HCl (pH 8.0)] were incubated for 24 h. Reactions were subsequently also performed with a 2-fold excess of fumarate. Fumarate (100 mM), amine (50 mM), and EDDS lyase (0.1 mg/mL) in buffer [Tris-HCl (pH 8.0)] were incubated for 24 h. All samples were prepared for LC–MS/MS analysis as follows. After incubation of the reaction mixture for 24 h, the samples were incubated at 100 °C for 1–2 min to stop the reaction. The precipitated enzyme was removed by centrifugation. The supernatant was filtered (0.42 μm filter) and subjected to LC–MS/MS to confirm formation of single- and/or double-addition products. Mass spectrometric analysis was performed by the Mass Spectrometry Facility Core in the Department of Pharmacy at the University of Groningen.

**Crystallization.** Before crystallization trials were set up, the EDDS lyase protein sample was further purified by gel filtration chromatography with a Superdex 200 (GE Healthcare) column using 0.2 M NaCl in 50 mM Tris-HCl buffer (pH 7.5) as an eluent. The protein eluted as a tetramer with an apparent molecular weight of ~200 kDa, as confirmed by dynamic light scattering analysis. The protein sample was concentrated to 9 mg/mL using a centrifugal concentrator (Vivaspin 15R, 30 kDa molecular weight cutoff, Sartorius Stedim Biotech). A search for crystallization conditions was performed using various commercial crystallization screens. Screening was performed at room temperature in 96-well sitting-drop crystallization plates using a robot (Mosquito, TTP LabTech) to dispense 300 nL drops (1:1 protein:reservoir ratio). A single cube-shaped crystal was obtained directly from the Structure Screen (Molecular Dimensions) with a solution containing 0.1 M HEPES (pH 7.5) and 4 M NaCl, but this condition could not be reproduced. Crystals also grew at a condition from Clear Screen II (Molecular Dimensions) with a solution containing 0.1 M HEPES (pH 7.5) and 40% PEG 400 and flash-cooled in liquid nitrogen. All the diffraction data were collected in house at 110 K using a Microstar rotating Cu anode X-ray source (Bruker AXS GmbH) in combination with Helios optics (Incoatec GmbH) and a MAR345dtb detector (MarResearch GmbH). Data sets were integrated and scaled using XDS and merged using the program AIMLESS from the CCP4 software suite. All crystals belonged to the F222 space group and contained a single polypeptide chain per asymmetric unit, with a solvent content of ~62%. Relevant data collection and refinement statistics are listed in Table S2. The upper resolution limit of the data sets varied between 2.6 and 1.9 Å. It should be noted that some of the data sets were recorded with a non-optimal crystal-to-detector distance, in which cases the actual resolution limit of the diffraction was somewhat higher. Structure determination was started with diffraction data collected from the single crystal obtained at 4 M NaCl. The program Phaser was used to obtain initial phases by molecular replacement. With the help of the Fold and Function Assignment Server (FFAS), an ensemble of three homologous protein structures was used as a molecular replacement search model: duck δ-crystallin II [28% identity, Protein Data Bank (PDB) entry 1TUJ], argininosuccinate lyase from *Thermus thermophilus* HB8 (30% identity, not published, PDB entry 2E9F), and duck δ-crystallin I (29% identity, PDB entry 1UI5). Automatic model building was performed using ARP/wARP. The programs REFMAC and COOT were used for subsequent rounds of refinement and model building, including the placement and validation of water molecules. The final rounds of refinement were performed using phenix.refine from the Phenix software suite. All other structures were adapted and refined starting from the apo structure, using identical strategies and software. Coordinates and restraints for formate, fumarate, and succinate were readily available from the CCP4 database with the following ligand identifiers, FMT, FUM, and SIN. Coordinates and restraints for (S,S)-EDDS and AEAA were generated using the PRODRG2 server (ENSUCP, CCDC no. 1149819).

**Crystal Structure Analysis.** Molprobity was used for validating the stereochemical quality of the models. Structure-based sequence alignments were performed using T-coffee and visualized using the ESPript 3.0 server. Superpositions and calculation of Ca backbone root-mean-square deviation (rmsd) values were performed using the protein structure comparison service Fold at the European Bioinformatics Institute. PyMOL (Schrödinger) was used for structure analysis and figure preparations. ChemBioDraw 12.0 was used to draw schemes and chemical structures. Atomic coordinates and structure factors have been deposited in the PDB (www.rcsb.org) as entries 6G3D, 6G3E, 6G3F, 6G3G, 6G3H, and 6G3I.

**RESULTS**

**Identification of an EDDS Lyase in *Chelativorans* sp. BNC1.** A sequence similarity search in the NCBI microbial database was performed using the BLASTP program using the EDDS lyase amino acid sequence from *Brevundimonas* sp. TN3 as the query. This search yielded several bacterial proteins that had sequences significantly similar to that of EDDS lyase. The top hits included a sequence from the bacterium *Chelativorans* sp. BNC1, which was isolated from industrial sewage receiving EDTA-containing wastewater effluents. This *Chelativorans* protein, with a sequence that is 79% identical to that of EDDS...
lyase from Brevundimonas sp. TN3 (Figure S1), was annotated as a putative argininosuccinate lyase and selected for further study.

The gene encoding the EDDS lyase homologue from Chelativorans sp. BNC1 was cloned into expression vector pBADN/Myc-His A, resulting in the construct pBADN(EDDS-His). Using this expression plasmid, the enzyme was produced upon induction with arabinose in E. coli TOP10 as a C-terminal hexahistidine fusion protein. The enzyme was purified by a one-step Ni-Sepharose affinity chromatography protocol, which typically provides \( \sim 30 \) mg of homogeneous enzyme per liter of culture. Analysis of the purified enzyme by size-exclusion chromatography and dynamic light scattering revealed a native molecular mass of \( \sim 200 \) kDa. A comparison of this value to that of the calculated subunit mass suggests that the enzyme is a homotetrameric protein.

To examine whether the Chelativorans enzyme can promote the synthesis of AEAA (3) and EDDS (1), the enzyme was incubated with fumarate (2) and ethylenediamine (4) and the reaction was monitored by \(^1\)H NMR spectroscopy (Figure 1). The results showed that the enzyme indeed catalyzes the addition of ethylenediamine to fumarate to give AEAA and \((S,S)\)-EDDS. The enzyme also catalyzes the reverse reaction, that is, the deamination of \((S,S)\)-EDDS to yield AEAA, fumarate, and ethylenediamine, as determined by UV and \(^1\)H NMR spectroscopy (data not shown). Having established that the Chelativorans enzyme exhibits EDDS lyase activity, we determined kinetic parameters (at 25 °C) and the optimum pH and temperature. The enzyme catalyzes the deamination of \((S,S)\)-EDDS with a \(k_{cat} \) of 6.5 ± 0.2 s\(^{-1}\) and a \(K_m\) of 16 ± 3 \(\mu M\) and shows maximum activity at pH 8.0 and 60 °C (Figure S2).

Notably, incubation of the enzyme with fumarate and arginine showed that the enzyme displays no argininosuccinate lyase activity.

**Substrate Scope of EDDS Lyase.** It has previously been determined that the Chelativorans enzyme accepts a wide variety of amino acids with terminal amino groups for selective addition to fumarate, yielding the natural products Aspergillomarasmine A and Aspergillomarasmine B, as well as various related aminocarboxylic acids (Scheme S1). To further explore the substrate scope of EDDS lyase, the enzyme was incubated with fumarate and different amines and the rate of addition was monitored by \(^1\)H NMR spectroscopy.
the reactions was monitored spectrophotometrically by following the depletion of fumarate at 240 nm. The results showed that EDDS lyase has a broad nucleophile scope and accepts various mono- and diamines as unnatural substrates in the amination of fumarate, albeit with a catalytic efficiency lower than that of the reaction with the native substrate ethylenediamine (Table 1). For several selected substrates, the enzymatic addition reactions were performed with either a 20-fold excess of amine or a 2-fold excess of fumarate, and formation of the corresponding single- and double-addition products was assessed by LC−MS/MS (Table S1). The results demonstrate that by using an appropriate molar ratio of starting substrates, different aminocarboxylic acid products, including EDDS derivatives, can be prepared using EDDS lyase as the catalyst. In contrast to its very broad nucleophile scope, the enzyme was found to be highly specific for fumarate, with fumaric acid monomethyl ester, crotonic acid, mesaconic acid, itaconic acid, 2-pentenoic acid, and glutaconic acid not accepted as alternative electrophiles.

Overall Structure of EDDS Lyase. The crystal structure of EDDS lyase was determined at 2.2 Å resolution by molecular replacement and refined to a crystallographic R-factor of 18.2% (Rfree = 22.2%) with good geometry (Table S2). Diffraction data were obtained from a single crystal grown in the presence of 4 M NaCl at pH 7.5. The EDDS lyase crystal structure belongs to space group F222 and contains one monomer per asymmetric unit (labeled A); the other three subunits (B−D) of the functional tetramer are “generated” by crystallographic 2-fold axes. The final model consists of 496 residues; only the first five residues at the N-terminus and the last seven residues at the C-terminus, which comprises Arg502 and the (His)6 tag, could not be modeled because of weak or absent electron density.

The overall fold and topology of EDDS lyase closely resemble those of other members of the aspartase/fumarase superfamily (Figures 2 and 3).12 The dumbbell-shaped, mainly α-helical subunit can be subdivided into three domains (Figure 2a): an N-terminal domain (residues 6−111), an elongated central domain (residues 112−351), and a C-terminal domain (residues 352−501). In the functional tetramer (Figure 2b), the central domains of the four subunits interact coaxially to form a tightly packed bundle of 20 α-helices, with the N- and C-terminal domains of neighboring subunits positioned near each other at the ends. Each active site (there are four in the tetramer) is composed of three regions of highly conserved amino acid residues, each of which originates from a different subunit (Figure S3 and Figure 2b). These conserved regions are C1 (residues 111−114; for a representative active site we will refer to the subunit as A), C2 (residues 158−165, subunit B), and C3 (also known as the “SS loop”, residues 279−293, subunit C).12

Two striking differences are observed between the overall structure of EDDS lyase and those of other aspartase/fumarase superfamily members (Figure 3). First, the EDDS lyase subunit contains a final C-terminal α-helix (residues 451−500) that is elongated (Figure S3) and runs alongside the entire length of the central domain back to the N-terminal domain. Second, within a subunit, the first ~30 residues at the N-terminus form a loop (residues 5−15) and a short α-helix (residues 20−31), which fold away from the N-terminal domain to pack against the central domain and SS loop. Two arginine residues (Arg10 and Arg13) in the N-terminal loop form hydrogen bonds with the main chain carbonyls of Ala274, Ala277, and Gln285, effectively locking the SS loop in place. In the tetramer, each SS loop is further stabilized by interactions with residues of the N- and C-terminal domains from two neighboring subunits, resulting in a rigid and well-defined conformation. In the structures of other aspartase/fumarase superfamily members, the SS loop is intrinsically flexible and can undergo an open−closed transition upon binding of substrate.12,34−37 In EDDS lyase, the occurrence of such a transition of the SS loop is unlikely: the additional interactions with the N-terminal loop region stabilize a conformation that is highly similar to the catalytically competent closed conformation observed in the

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</tr>
<tr>
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<td>NH₂</td>
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<tr>
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<tr>
<td>20</td>
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<td>NH₂</td>
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*The initial rate of the enzyme-catalyzed addition of ethylenediamine to fumarate was assigned as 100% activity.
structures of other aspartase/fumarase superfamily members, even when no substrate is bound.

**Formate-, Fumarate-, and Succinate-Bound Structures.** During the initial crystallization screening, a condition that included 2.0 M sodium formate (pH 6.5) also yielded protein crystals. The crystals belonged to the same space group, F222, obtained with 4 M NaCl, with identical cell parameters, and allowed a structure determination at 1.9 Å resolution. The overall structure of EDDS lyase in the crystals grown with sodium formate is identical to that of the crystal obtained with sodium chloride, including the conformation of the SS loop (the structures superpose with an rmsd value of only 0.26 Å).
Interestingly, however, extra wedge-shaped electron density clearly indicated the presence of three formate ions bound in the active site (Figure 4a and Figure S4a). One formate ion (FMT1) makes hydrogen bonds to Ser111 and Arg112 (C1 region) from subunit A and Ser281 (SS loop) from subunit C, while another formate ion (FMT2) makes hydrogen bonds to Asn113 (C1 region) from subunit A, Thr158 (C2 region) from subunit B, and Lys286 and Asn288 (SS loop) from subunit C. The third formate ion (FMT3) is bound farther from the SS loop making an interaction with Arg112. On the basis of a comparison with other aspartase/fumarase superfamily structures, it was realized that two of these formate ions (FMT1 and FMT2) likely mimic the carboxylate groups of the succinyl moiety of the substrate, or of the product fumarate, when bound to the active site. To investigate this further, we repeated the crystallization experiments using solutions in which sodium formate was substituted with sodium salts of fumaric acid or succinic acid. Large cube-shaped crystals appeared overnight under conditions including a 0.2−0.3 M concentration of either salt, allowing the structural determination of fumarate-bound and succinate-bound EDDS lyase at 2.2 and 2.6 Å resolution, respectively (Table S2, Figure 4b,c, and Figure S4b,c). The fumarate and succinate molecules are tightly coordinated at the active site with overall identical binding interactions. As expected, the C1 and C4 carboxylate groups of fumarate/succinate occupy the same positions as formate ions FMT1 and FMT2 discussed above, making similar hydrogen bonds with residues from the C1, C2, and SS loop region. Most notably, the hydroxyl group of Ser280 in the SS loop (subunit C) is positioned close to (−3 Å) and in a proper orientation from the Cβ atom in fumarate/succinate, in accordance with its presumed role as a catalytic base in the α,β-elimination reaction.

EDDS- and AEAA-Bound Structures. Using an approach similar to that described above, crystals diffracting to 2.2 Å resolution were obtained of EDDS lyase grown in the presence of 0.3 M (S,S)-EDDS. Because the enzyme can convert (S,S)-EDDS to fumarate and ethylenediamine, we expected to observe a fumarate ion bound at the active site. Much to our surprise, however, the electron density at the active site was consistent with the presence of an intact (S,S)-EDDS molecule, allowing the determination of the substrate-bound structure (Table S2 and Figures 4d and 5). Apparently, the conditions for cleavage of (S,S)-EDDS in the crystal are not ideal, which may be explained by the non-optimal pH (6.5) of the crystallization solution (optimal pH for activity is 8) and/or insufficient protein flexibility preventing the deamination step and effectively trapping (S,S)-EDDS in the active site. One of the two succinyl moieties of (S,S)-EDDS (we refer to it as the proximal succinyl, on the basis of its proximity to the SS loop) binds at the active site like fumarate or succinate does in the other EDDS lyase structures, making identical interactions with the C1 and C2 regions and the SS loop (Figure 6). The other (distal) succinyl moiety points away from the active site and is bound near the protein surface, with one of the carboxylate groups forming hydrogen bonds with residues Arg294 and Tyr26 from chain C of the functional tetramer. The other largely solvent exposed carboxylate group makes a water-mediated hydrogen bond with Asn113. A comparison of the formate- and EDDS-bound structures shows that although the third formate ion (FMT3) and the carboxylate group of the distal succinyl moiety bind at similar regions of the active site pocket, their actual binding site locations and geometries are quite different (see Figure 4a,d). Thus, unlike FMT1 and FMT2, FMT3 indeed does not mimic the binding of one of the carboxylates of (S,S)-EDDS. In addition, the internal amino
group of (S,S)-EDDS that is directly linked to the proximal succinyl moiety forms a hydrogen bond with Asn113, while the other amino group linked to the distal succinyl moiety makes water-mediated hydrogen bonds to Asn288 and Asp290.

To obtain a full description of all relevant structures in the catalytic cycle of EDDS lyase, crystals were also grown in the presence of the reaction intermediate AEAA. Surprisingly, instead of AEAA, a molecule of (S,S)-EDDS was found in the active site bound in an identical conformation as in the structure obtained from a crystal grown in the presence of (S,S)-EDDS (data not shown). A possible explanation for this result is that EDDS lyase in solution will reversibly cleave AEAA to fumarate and ethylenediamine. EDDS lyase likely first crystallizes in a fumarate-bound state, after which (S,S)-EDDS can be produced in the crystal in one step by attack of AEAA. This can happen as long as the concentration of AEAA in the crystallization solution is sufficiently high. Upon formation of (S,S)-EDDS in the crystal, it will remain trapped in the active site [as shown by the co-crystallization with (S,S)-EDDS]. In another attempt to obtain an AEAA-bound structure, a fumarate-bound crystal (prepared by crystallizing the enzyme in the presence of 0.3 M fumarate) was briefly soaked for \(\sim 30\) s in mother liquor containing 20 mM ethylenediamine. The crystal was then immediately flash-cooled in liquid nitrogen and used for X-ray diffraction data collection. Difference Fourier analysis of the active site revealed a small adduct at the C\(\alpha\) atom of bound fumarate, most likely resulting from the addition of ethylenediamine to form the intermediate AEAA. Subsequent refinement yielded an AEAA-bound structure at 2.4 Å resolution (Table S2, Figure 4e, and Figure S4d). It should be noted that in this structure the ethylenediamine moiety of the bound AEAA molecule is relatively disordered, as indicated by

![Figure 5](image-url)
the absence of electron density for the terminal, free amino group. In addition, a second molecule of fumarate is observed near AEAA, bound at a location similar to where the distal succinyl moiety of \((S,S)\)-EDDS binds. Possibly, this second molecule of fumarate prevented a quick release of AEAA, thereby inhibiting the full conversion toward \((S,S)\)-EDDS.

**Mutagenesis of Active Site Residues.** The crystal structure of the enzyme in complex with \((S,S)\)-EDDS (Figure S5) suggests important roles for Ser280 and Asp290 in catalysis and substrate binding, respectively. Ser280 is positioned near the Cβ proton of the substrate and in a suitable orientation to allow proton abstraction to initiate the deamination reaction. Asp290 forms a water-mediated hydrogen bond with the internal amino group connected to the distal succinyl moiety of \((S,S)\)-EDDS, which appears to be an important interaction for binding and positioning of ethylenediamine for addition to fumarate. To study the importance of these residues for catalytic activity, each residue was replaced with an alanine. The mutation of Ser280 to an alanine resulted in an inactive enzyme (data not shown), confirming the essential role of this residue in catalysis. The mutation of Asp290 to an alanine resulted in a mutant enzyme that displayed activity significantly lower than that of wild-type EDDS lyase for the addition of ethylenediamine to fumarate (Figure S5).

**DISCUSSION**

The crystal structures of EDDS lyase determined in this study confirm a tertiary and quaternary fold that is characteristic of the aspartase/fumarase superfamily and provide the first detailed view of the substrate- and product-bound active site of an EDDS lyase, allowing an understanding of the roles of the various active site residues in substrate binding and catalysis (Figure 7). In all members of the aspartase/fumarase superfamily, a strictly conserved serine residue from the SS loop has been implicated to act as the catalytic base, abstracting a proton from the Cβ atom of the substrate to initiate the αβ-elimination reaction.12,34−37 In EDDS lyase, the equivalent SS loop serine residue, Ser280, most likely functions as the catalytic base. Indeed, in both the \((S,S)\)-EDDS-bound and the

![Figure 6](image1.png)

**Figure 6.** Superposition of bound \((S,S)\)-EDDS (wheat), fumarate (light blue), and succinate (pale cyan) in the active site of EDDS lyase. The SS loops of the \((S,S)\)-EDDS-bound, fumarate-bound, and succinate-bound structures, with putative catalytic base Ser280, are colored green, yellow, and magenta, respectively. The distance in angstroms from the hydroxyl group of Ser280 to the Cβ atom of the \((S,S)\)-EDDS substrate is shown as a dashed line.

![Figure 7](image2.png)

**Figure 7.** Proposed catalytic mechanism for the deamination of \((S,S)\)-EDDS to yield AEAA and fumarate.

AEAA-bound structures, Ser280 is positioned near (~3 Å) the Cβ proton of the substrate (Figures 5, 6 and S4d) and in a suitable orientation to allow proton abstraction in the first step of the reaction (Figure 7). As expected, mutation of Ser280 to an alanine resulted in an inactive enzyme. However, for Ser280 to function as the catalytic base, it needs to be activated to form a Ser-O⁻ oxyanion. Possible mechanisms for activation of the catalytic serine in aspartase/fumarase superfamily members have been suggested previously, including substrate-assisted deprotonation of the serine residue to generate the Ser-O⁻ oxyanion.12 The serine oxyanion could be stabilized by main chain amide interactions. In the structure of EDDS lyase, Ser280 is within hydrogen bonding distance of the neighboring main chain backbone amides of Ser281, Ile282, and Met283, which form part of the SS loop. The question of how Ser280 is activated to function as the catalytic base thus remains to be answered.
The extensive number of hydrogen bonding interactions observed for the binding of (SS)-EDDS and AEAA (Figures S5 and S4d) forces the substrates to adopt an energetically unfavorable rotamer conformation in which the $\alpha\,\beta$, $\beta\,\alpha$, and $\beta\,\beta$-carboxylic atoms are coplanar. This is consistent with a conformation resembling a putative enediolate (acylcarboxylate) intermediate during catalysis (Figure 7).

The oxygens of the $\beta$-carboxylate group of (SS)-EDDS and AEAA form hydrogen bonds with the side chain hydroxyls of Ser111 (subunit A) and Ser281 (subunit C, SS loop), the guanidinium moiety of Arg112 (subunit A), and main chain amides of Arg112 and Ser281. This extensive hydrogen bonding network not only is essential for substrate binding but also plays a crucial role in stabilizing the additional negative charge that develops on one of the $\beta$-carboxylate oxygens as a result of $\alpha\,\beta$ proton abstraction by Ser280. The presence of a positively charged residue, Arg112, has an additional stabilizing effect on the negatively charged $\alpha\,\beta$-carboxylate intermediate. Both (SS)-EDDS and AEAA are expected to employ similar catalytic mechanisms for the cleavage of the C–N bond (Figure 7). Hence, our results, combined with earlier mechanistic studies of mechanisms for the cleavage of the C–EDDS and AEAA are expected to employ similar catalytic on one of the $\beta$-carboxylate oxygens as a result of $\alpha\,\beta$ proton abstraction by Ser280. The presence of a positively charged residue, Arg112, has an additional stabilizing effect on the negatively charged $\alpha\,\beta$-carboxylate intermediate. Both (SS)-EDDS and AEAA are expected to employ similar catalytic mechanisms for the cleavage of the C–N bond (Figure 7). Hence, our results, combined with earlier mechanistic studies of mechanisms for the cleavage of the C–EDDS and AEAA are expected to employ similar catalytic on one of the $\beta$-carboxylate oxygens as a result of $\alpha\,\beta$ proton abstraction by Ser280. The presence of a positively charged residue, Arg112, has an additional stabilizing effect on the negatively charged $\alpha\,\beta$-carboxylate intermediate. Both (SS)-EDDS and AEAA are expected to employ similar catalytic mechanisms for the cleavage of the C–N bond (Figure 7). Hence, our results, combined with earlier mechanistic studies of mechanisms for the cleavage of the C–EDDS and AEAA are expected to employ similar catalytic.
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**REFERENCES**


