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Purification and characterization of a lyase from the EDTA-degrading bacterial strain DSM 9103 that catalyzes the splitting of [S,S]-ethylenediaminedisuccinate, a structural isomer of EDTA

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Abstract

The bacterial strain DSM 9103, able to utilize EDTA as a sole source of carbon, nitrogen, and energy, is also capable to grow with [S,S]-ethylenediaminedisuccinate ([S,S]-EDDS), a structural isomer of EDTA. In cell-free extracts of [S,S]-EDDS-grown bacteria, [S,S]-EDDS degradation was observed in the absence of any cofactors. An enzyme was purified 41-fold that catalyzed the non-hydrolytic splitting of [S,S]-EDDS leading to the formation of fumarate and N-(2-aminoethyl) aspartic acid. These data strongly suggest that the enzyme belongs to the group of carbon-nitrogen lyases. The splitting reaction was reversible, and an equilibrium constant of approximately 43.0 10^{-1} M was determined. Out of the three stereo-isomers of EDDS, [S,S]- and [R,S]-EDDS were accepted as substrates by the lyase, whereas [R,R]-EDDS remained unchanged in assays with both cell-free extracts and pure enzyme. The enzyme catalyzed the transformation of free [S,S]-EDDS and of [S,S]-EDDS-metal complexes with stability constant lower than 10, namely of MgEDDS, CaEDDS, BaEDDS and to a small extent also of MnEDDS; Fe_{III}EDDS, NiEDDS, CuEDDS, CoEDDS and ZnEDDS were not transformed.

Abbreviations: AEAA – N-(2-aminoethyl) aspartic acid; EDDS – ethylenediaminedisuccinate; EDTA – ethylenediaminetetraacetate; NTA – nitrilotriacetate

Introduction

Strong chelating agents are extensively used in a wide variety of industrial applications to inactivate free metal ions. Aminopolycarboxylic acids and their salts are by far the dominant group of substances used as chelating agents worldwide. Among these, ethylenediaminetetraacetate (EDTA) is the most frequently employed compound because of its advantageous physical and chemical properties (Egli 1988). Because most of its applications are water based, a large proportion of the EDTA is released into wastewater treatment plants or the aqueous environment. EDTA, however, is poorly biodegradable (Alder et al. 1990; Wolf & Gilbert 1992), and presently the only known significant sink of EDTA in the environment is the photolysis of the photolabile species Fe_{III}EDTA by sunlight (Wolf & Gilbert 1992; Kari 1994). Consequently, its ubiquitious distribution in both surface and groundwaters was demonstrated (Houriet 1996; Frimmel et al. 1989; Dietz 1987). Because further accumulation of EDTA might lead to a mobilization of heavy metals in sediments and infiltration areas (Frimmel et al. 1989; Nowack 1996), it is of high interest to develop alternative chelating agents which possess good complexing properties and are easily degradable in the environment.

In this respect, ethylenediaminedisuccinate (EDDS), a structural isomer of EDTA, appears to be a promising compound (Hartman & Perkins 1987; Kovaleva

et al. 1992). The molecule is made up of two fragments of the amino acid aspartate. Thus, it contains two chiral carbon atoms resulting in the existence of three optical isomers, [S,S]-EDDS, [R,R]-EDDS and [R,S]-EDDS. [S,S]-EDDS is a naturally occurring compound synthesized by the actinomycete *Amycolatopsis orientalis* (Nishikiori et al. 1984; Cebulla 1995). It is thought to be involved in a specific zinc uptake system because [S,S]-EDDS is only produced under zinc-limited growth conditions (Cebulla 1995). Two reactions for the chemical synthesis of EDDS were described: The reaction of maleic acid or maleic anhydride and ethylenediamine yields a mixture of three optical isomers (Gorelov et al. 1979; Hartman 1987), whereas either pure [S,S]-EDDS or [R,R]-EDDS is obtained by a reaction of aspartic acid with 1,2-dibromoethane, depending on the stereo-isomer of the aspartic acid employed (Neal & Rose, 1968)

The degradability of the stereo-isomers of EDDS was tested with activated sludge from different sources (Schowanek et al. 1997). In all cases, only [S,S]-EDDS was rapidly and completely mineralized, whereas biodegradation of [R,R]-EDDS was negligible. Degradation of the R,S-isomer was found to lead to the formation of the recalcitrant intermediate N-(2-aminoethyl) aspartic acid (AEAA) probably present in the D-configuration (Schowanek et al. 1997). These observations suggest that [S,S]-EDDS should be favoured for the application in both domestic and industrial products.

Besides the biodegradation tests with activated sludge, no studies on the microbial breakdown of EDDS have been reported up to now. We tested several nitrilotriacetate (NTA)- or EDTA-degrading bacterial strains that had previously been isolated in our laboratory (Egli et al. 1988; Witschel et al. 1997) for their ability to grow with [S,S]-EDDS as sole source of carbon, nitrogen, and energy. Two strains, the NTA-degrader *Chelatococcus asaccharovorans* (Auling et al. 1993) and the EDTA-degrader DSM 9103 (Witschel et al., 1997) exhibited growth with this compound. Furthermore, we isolated another [S,S]-EDDS-degrading bacterium designated strain E1 from activated sludge of the municipal wastewater treatment plant Duebendorf. In the present paper the first step of [S,S]-EDDS catabolic pathway in these strains, especially in the EDTA-degrader DSM 9103, is described.

Materials and methods

Chemicals

[S,S]-EDDS was obtained from the laboratory of H. Zähner (University of Tübingen, Germany), from Ciba Chemikalien GmbH (Grenzach-Wyhlen, Germany) and from The Procter and Gamble Company (Cincinnati, USA). [R,R]-EDDS and EDDS enantiomeric mixture containing 25% [S,S]-EDDS, 25% [R,R]-EDDS and 50% [R,S]-EDDS as well as AEAA were gifts of The Procter and Gamble Company. Argininosuccinate was purchased from Sigma (St. Louis, USA). All other chemicals were of analytical grade and were obtained from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

Growth of microorganisms

The strains DSM 9103, *C. asaccharovorans* and strain E1 were grown at 30 °C on a mineral medium without a nitrogen source (Egli et al. 1988) containing approximately 1 g L^{-1} of [S,S]-EDDS as sole source of carbon, nitrogen, and energy. For the purification of the [S,S]-EDDS-degrading enzyme from DSM 9103, high amounts of biomass were produced by growing the strain in an aerated 20 L batch culture with the above described medium. Ortho-phosphoric acid was used to control the pH within a range of 7.0 to 7.5. At an OD_{546} of 1.3, the cells were harvested by centrifugation and then washed once with 0.05 M Tris/HCl (pH 7.0). The obtained cell paste (approximately 40 g wet weight) was stored at − 20 °C.

Preparation of cell-free extract

20 g wet weight of frozen cell paste was thawed at room temperature and suspended in 30 ml of 0.05 M Tris/HCl (pH 7.0). After homogenisation for 1 min at medium speed with a Polytron homogenisator (Kinematic, Luzern, Switzerland), 10 mg of DNaseI (Fluka, Buchs, Switzerland) was added. Then the cells were broken by three passages through a French press (Aminco, Urbana, USA) at 20000 psi. After each passage, the suspension was cooled down to 0 °C by stirring it on ice for 10 min. The crude extract was centrifuged for 30 min at 15000 g to remove unbroken cells and cell debris. Subsequently, the membrane fraction was removed by centrifugation at 160000 *g* for 1.5 h. The resulting cell-free extract was used for enzyme assays and protein purification.

Purification of the [S,S]-EDDS-degrading enzyme

A first purification was obtained by ammonium sulfate fractionation. At 0 °C, the fraction containing the [S,S]-EDDS-degrading activity precipitated at 40% saturation with ammonium sulfate and was collected by centrifugation (3000 *g*, 40 min). The resulting pellet was resuspended in 0.02 M Tris/HCl (pH 8.0) using approximately twice the volume of the pellet. This solution was desalted by diafiltration with Centricon-30 tubes (Amicon, Beverly, USA) and then chromatographed on an anion exchange column [Fractogel®EMD TMAE 650 (S), 10 by 150 mm; Merck] equilibrated with 0.02 M Tris/HCl (pH 8.0) at a flow rate of 1 ml min^{-1}. After loading the protein solution, the column was washed with the equilibration buffer containing 0.1 M NaCl. The fraction containing [S,S]-EDDS-degrading activity was eluted with a Tris/HCl buffer (pH 8.0) containing 0.15 M NaCl. After diafiltration, the protein solution was loaded on a phenyl sepharose column (High Load Phenyl Sepharose® High Performance, 10 by 160 mm; Pharmacia, Uppsala, Sweden) equilibrated with 0.04 M Tris/HCl (pH 7.0), 0.5 M $(NH_4)_2SO_4$ at a flow rate of 1.0 ml min^{-1}. After washing the column with a linear gradient from 0.5 to 0.1 M ammonium sulfate within 15 min, the [S,S]-EDDS-degrading enzyme was eluted using a Tris-HCl buffer (pH 7.0) containing 0.05 M ammonium sulfate.

Protein determination

Protein concentrations were measured according to the method described by Bradford (1976). Bovine serum albumine (Fluka) was used as standard.

Enzyme assay

Enzyme assays with cell-free extracts or the purified enzyme were performed at 30 °C in 0.05 M Tris/HCl buffer (pH 7.5) In some cases, 0.3 μmol min^{-1} ml^{-1} of fumarase (Sigma) was also added to the reaction mixture. The reaction was started by the addition of EDDS, and aliquots of 100 μl were withdrawn as function of time. The samples were boiled in order to stop the reaction. Precipitated proteins were removed by centrifugation at 20000 *g* for 5 min, and the supernatant was then analysed for EDDS, fumarate, and malate as described below.

Analysis of EDDS

[S,S]-EDDS was measured as Fe_{III}-complex by HPLC with a Lichrocart®250-4 C18 column (Merck) following the same method as applied for the determination of EDTA (Witschel et al. 1997). A formate buffer (5 mM sodium formate, 15 mM formic acid, 2 mM tetrabutylammoniumbromide) containing 12.5% methanol was employed as the eluent. For the analysis of [R,R]-EDDS and the EDDS enantiomeric mixture, the same colum and eluent were used, but copper complexes were formed by diluting the samples 1 : 10 with a buffer containing 3 mM copper acetate and 10 mM t-butylammonium hydroxide; pH adjusted to 3 with phosphoric acid. Under these conditions, [R,S]-EDDS could be separated from [R,R]- and [S,S]-EDDS and retention times of 11.5 min and 16.7 min were observed for [R,S]-EDDS and [R,R]/[S,S]-EDDS respectively.

Analysis of fumarate and malate

Both dicarboxylic acids were analysed by HPLC with a HPICE-AS1 ion exclusion column (Dionex, Sunnyvale, USA) as described by Schneider and coworkers (1988).

Analytical gelfiltration

The native weight of the [S,S]-EDDS-degrading enzyme was determined by analytical gelfiltration on a Superose 6 HR (10 by 300 mm) column (Pharmacia). 0.05 M Tris/HCl (pH 7.5) containing 0.1 M NaCl was the eluent. With the help of the Gelfiltration Calibration Kit (Pharmacia) for low and high molecular weight proteins the calibration curve was established.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous gel electrophoresis was performed following the protocol of Lämmli (1970) using 12% SDS-Polyacrylamide separating gels. Molecular weight determination of the denatured enzyme was done with the help of the Low Molecular Weight Calibration Kit of Pharmacia. The gels were stained with Coomassie Brilliant Blue G 250.

Table 1. Purification of EDDS-lyase from the bacterial strain DSM 9103

Purification step	Vol (ml)	Total amt (mg) of proteins	Sp act[a] (μmol/min/mg)	Recovery (%)	Purification (fold)
Cell-free extract	27.5	836	0.84	100	1
Ultracentrifugation	25.6	586	1.36	114	1.6
Ammonium sulfate (40% saturation)	5.3	128	4.4	80	5.2
TMAE Fractogel	4.3	26	14.1	52	16.8
Phenyl Sepharose	1.4	12	34.1	58	40.6

[a] The specific activity was determined by measuring the disappearance of [S,S]-EDDS in the enzyme assay.

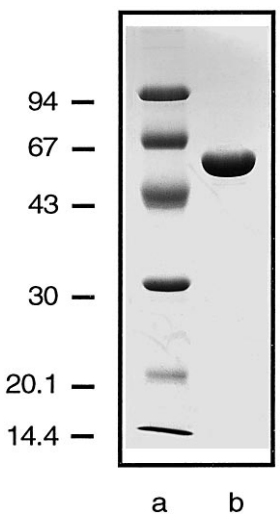

Figure 1. SDS-polyacrylamid gel electrophoresis of EDDS-lyase. Lane a, standard marker proteins; lane b, EDDS-lyase.

Results

Degradation of [S,S]-EDDS in cell-free extracts

Cell-free extracts prepared from cells of the bacterial strain DSM 9103 grown with [S,S]-EDDS as sole source of carbon, nitrogen, and energy exhibited [S,S]-EDDS-consuming activity in the absence of any added cofactor. Degradation of [S,S]-EDDS led to the formation of fumarate and malate. Fumarase activity, which catalyzes the reversible hydration-dehydration of fumarate and L-malate, was detected in cell-free extracts suggesting that only one of the two bicarboxylic acids detected was a direct product of [S,S]-EDDS breakdown, whereas the other was the product of a subsequent reaction.

In cell-free extracts prepared from [S,S]-EDDS-grown cells of either *C. asaccharovorans* or strain E1, the formation of the same two products from [S,S]-EDDS was found also without requirement of any cofactors. This suggested that the same type of enzymatic reaction was responsible for the degradation of [S,S]-EDDS in all three bacterial strains.

Cell-free extracts of DSM 9103 grown with EDTA or fumarate plus NH_4^+ as sole source of carbon, nitrogen, and energy had no [S,S]-EDDS-degrading activity at all. In cell-free extracts of NTA-grown *C. asaccharovorans*, no consumption of [S,S]-EDDS was observed either.

Purification and characterization of the [S,S]-EDDS-degrading enzyme

By a procedure based on ammonium sulfate precipitation, anion exchange, and hydrophobic interaction chromatography, a 40.6-fold purification of the [S,S]-EDDS-degrading protein from cell-free extracts of DSM 9103 with an overall yield of 58% was achieved (Table 1).

The pure enzyme exhibited a typical protein absorption spectrum with a maximum at 280 nm and no further maxima in the region between 300 and 700 nm. The apparent molecular weight of the enzyme determined by gelfiltration was 130 kD. SDS-PAGE revealed one band corresponding to a molecular weight of 58 kD (Figure 1) indicating that the enzyme is a homodimer.

Characterization of the enzymatic activity of the pure protein

The pure enzyme degraded [S,S]-EDDS with the formation of fumarate plus an unknown product. Again, no cofactors had to be added for the reaction to take place. When fumarase was included in the enzyme assay, both fumarate and malate were observed as prod-

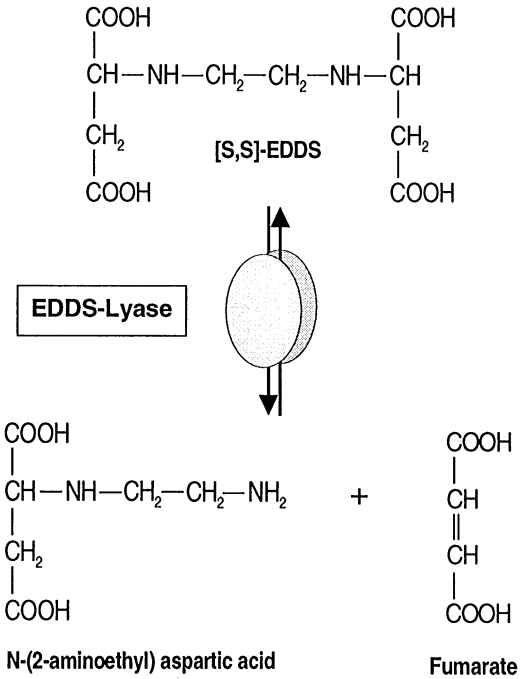

Figure 2. Reversible reaction catalyzed by the EDDS-lyase in the EDTA-degrading bacterial strain DSM 9103.

ucts of the reaction. These results demonstrate that the [S,S]-EDDS-degrading enzyme is a lyase catalysing an elimination reaction by splitting a C-N-bond between one succinyl residue and the ethylenediamine part of the molecule (Figure 2). Hence, one can assume that AEAA was the second product of the reaction. Maleic acid, the isomer of fumarate with a cis-configuration of the double bond instead of a trans-configuration, could not be detected as product of the [S,S]-EDDS-degradation.

The pH optimum for lyase activity was at pH 7.5. The optimum temperature for the enzyme-catalysed reaction was rather high, with maximum activities observed between 45 and 50 °C. A K_m-value of 0.2 ± 0.02 mM [S,S]-EDDS was found for the lyase. By using equimolar concentrations of different metal ions and [S,S]-EDDS, it was tested which species of metal-complexed [S,S]-EDDS were transformed by the lyase. Besides free [S,S]-EDDS, the complexes of [S,S]-EDDS with earth alkaline metals were readily degraded. The specific activities with free [S,S]-EDDS and with complexes of these metals were almost identical. Furthermore, MnEDDS was consumed but with a low specific activity (approximately 14% of the specific activity with free [S,S]-EDDS) and all other species analyzed (ZnEDDS, CoEDDS, NiEDDS, CuEDDS and Fe_{III}EDDS) were not transformed at all.

In addition, it was investigated whether EDDS-lyase has similarities to presently known C-N lyases from the amino acid metabolism and accepts either aspartate or argininosuccinate as substrate. The transformation of these two compounds results in the formation of fumarate. In both cases, no fumarate production was detected by purified EDDS-lyase. Furthermore, it was tested whether or not AEAA can serve as a substrate for EDDS-lyase leading to a second splitting reaction with fumarate and ethylenediamine being formed. However, neither in the presence of the purified enzyme nor of cell-free extract was a significant fumarate formation observed when AEAA replaced [S,S]-EDDS in the enzyme assay.

Back-reaction and equilibrium

The non-hydrolytic cleavage of [S,S]-EDDS catalyzed by EDDS-lyase is freely reversible, as was demonstrated by offering AEAA and fumarate as substrates for the enzyme. Under these conditions, the formation of EDDS was demonstrated. Since a racemic mixture of D- and L-AEAA was used to analyze the formation reaction it was possible that all three isomers were formed. While [R,S]-EDDS was definitely produced, it is not clear whether [S,S]-EDDS or [R,R]-EDDS or both were produced because the latter two could not be distinguished by the analytical tools at hand. Nevertheless, the substrate specificity of the lyase catalyzing only the degradation of [S,S]- and [R,S]-EDDS (see below) suggests that [S,S]- and no [R,R]-EDDS was formed in the back-reaction. When in the back-reaction fumarate was replaced by maleic acid very small amounts (approximately 15 times less than in the case of fumarate being an educt) of [R,S]- and [R,R]/[S,S]-EDDS were formed.

As the reaction is reversible, an equilibrium constant was calculated according to the expression:

$$K_{eq.} = \frac{[AEAA][fumarate]}{[EDDS]}$$

with [AEAA], [fumarate], and [EDDS] being the concentrations of the substances at equilibrium conditions. In preliminary experiments, it was found that the equilibrium was normally reached after 15 to 20 min. Thus, equilibrium concentrations of substrate and products were determined after 30 min incubation time at 30 °C with 2.5 μl lyase per ml. Whereas both [S,S]-EDDS and fumarate concentrations were

Table 2. Determination of the equilibrium constant of the EDDS-lyase catalyzed reaction at constant intial [S,S]-EDDS concentrations and varying initial fumarate concentrations

Initial concentration (mM)		Final concentration (mM)		$K_{eq.}$ (mM)
[S,S]-EDDS	Fumarate	[S,S]-EDDS	Fumarate	
3.6	0	0.24	3.05	42.7
3.54	1.01	0.32	4.11	41.4
3.54	1.89	0.35	4.54	41.4
3.77	3.99	0.47	6.33	44.4
3.88	8.03	0.73	10.49	45.3
Average				43.0

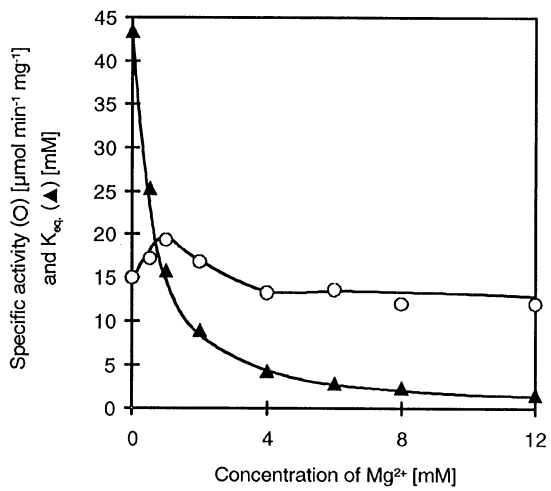

Figure 3. Influence of different Mg^{2+} concentrations on the specific activity of the EDDS degradation catalyzed by purified EDDS-lyase and on the equilibrium constant ($K_{eq.}$) of the reaction.

measured before and after incubation, the equilibrium concentration of AEAA was calculated from the amount of [S,S]-EDDS that had disappeared. The assay was performed at a constant initial [S,S]-EDDS concentration (approximately 3.7 mM) and by varying the initial fumarate concentration (0, 1, 2, 4, and 8 mM of fumarate). An equilibrium constant of $43.0 \cdot 10^{-1}$ M was calculated from the average of the values obtained at different intial fumarate concentrations (Table 2). The change in free energy, ΔG_0, calculated from this value is 7.9 kJ mol^{-1}. It is in accordance with the ready reversibility of the reaction observed experimentally.

When the back-reaction was performed in the presence of Mg^{2+} (12 mM) more [S,S]- or [R,R]-EDDS was formed compared to assays containing no Mg^{2+}. The amount of [R,S]-EDDS, however, was only slightly higher in the presence of Mg^{2+} than in its absence. Furthermore, the back-reaction was analyzed in the presence of Cu^{2+} ions with copper concentrations varying between 0 and 15 mM. For the highest concentrations tested (12 and 15 mM Cu^{2+}), no significant EDDS formation was detected. In the case of Cu^{2+}-concentrations equal to or lower than 8 mM, the amount of EDDS formed increased with increasing Cu^{2+} concentrations. Whereas in the absence of Cu^{2+} and for a Cu^{2+} concentration of only 1 mM the amount of [R,S]-EDDS and [R,R]/[S,S]-EDDS produced was nearly equal, there was always more [R,R]/[S,S]-EDDS than [R,S]-EDDs formed for all higher Cu^{2+} concentrations. These results suggested that metal ions have an influence on the equilibrium of the EDDS-lyase catalyzed reaction. Therefore, both the initial reaction rates of EDDS-degradation and the equilibrium constant were measured in the presence of a constant initial [S,S]-EDDS concentration of 4 mM and different Mg^{2+} concentrations. Figure 3 shows that the initial reaction rates did not change significantly with increasing Mg^{2+} concentrations, whereas the equilibrium constant decreased from $43 \cdot 10^{-3}$ M in the absence of Mg^{2+} to $1.6\ 10^{-3}$ M at 12 mM Mg^{2+}. When Mg^{2+} was replaced by Ca^{2+}, a similar pattern was observed. While the initial velocity was roughly the same for the different Ca^{2+} concentrations tested, the equilibrium constant decreased, although the decrease was much less pronounced than with Mg^{2+}. Because of this difference, the influence on the equilibrium constant of earth alkaline metal ions was analyzed by supplying metal ions in stoichiometric amounts to the initial [S,S]-EDDS concentration into the enzyme assay. In the presence of Mg^{2+}, the equilibrium constant was by a factor of 10 lower than in the absence of Mg^{2+}. For Ca^{2+} and Ba^{2+}, how-

ever, they were reduced only by a factor of 2 and 1.3 respectively.

When EDTA (10 mM) was added to the reaction mixture, no influence on the inital EDDS degradation rate nor on the equilibrium constant was seen. In a further experiment, the reaction mixture initially contained EDDS-lyase, 4 mM [S,S]-EDDS, 10 mM EDTA and 10 mM or 14 mM Mg^{2+}. Based on the stability constants of the Mg^{2+}-complexes of [S,S]-EDDS and EDTA (Martell & Smith 1974), the initial concentrations of MgEDDS were calculated and values of 0.56 mM and 3.92 mM respectively were determined. Again the initial EDDS degradation rate remained unchanged in both cases, but the equilibrium constants observed were lower than in the absence of Mg^{2+}. They were similar to the values obtained in the case of the enzymatic [S,S]-EDDS degradation in the presence of 0.5 mM Mg^{2+} (calculated MgEDDS concentration = 0.4998 mM) and of 4 mM Mg^{2+} (calculated MgEDDS concentration = 3.86 mM) respectively).

Furthermore, it was tested whether or not EDDS-lyase would also catalyze the formation of EDDS from ethylenediamine and two molecules of fumarate. Under the chosen assay conditions EDDS formation from AEAA and fumarate had already been shown. In order to shift the equilibrium towards EDDS, 5 mM Cu^{2+} were added to the assay mixture. The initial concentrations of ethylenediamine and of fumarate were 10 mM and 20 mM, respectively. However, no EDDS production was observed from ethylenediamine within 1 h.

Degradation of different enantiomers of EDDS by whole cells, cell-free extract and EDDS-lyase

Cells of the strain DSM 9103 grew on [S,S]-EDDS with a maximum growth rate of μ_{max} = 0.075 h^{-1}. The strain also grew on the enantiomeric mixture containing 25% [S,S]-EDDS, 25% [R,R]-EDDS, and 50% [R,S]-EDDS. In this case, the [R,S] isomer was consumed by DSM 9103 nearly totally, whereas roughly one half of the amount of [R,R]- and [S,S]-EDDS – which could not be distinguished analytically – remained in the growth medium. Disapperance of the isomers took place simultaneously and at approximately the same rate. Only for pure [R,R]-EDDS neither growth nor utilization of the substance was observed. Therefore, one can assume that from an enantiomeric mixture, [R,S]- and [S,S]-EDDS were consumed, whilst the R,R-isomer remained unaffected.

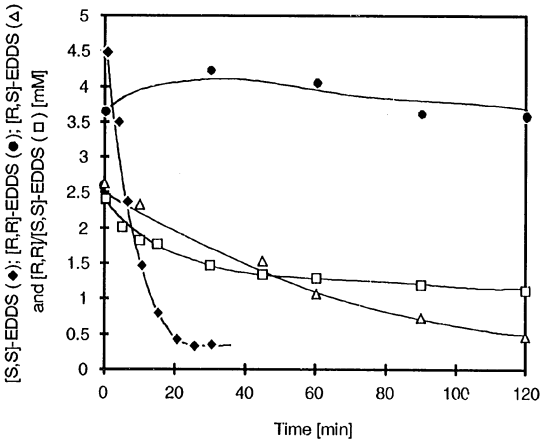

Figure 4. Lyase-catalyzed degradation of pure [S,S]- and [R,R]-EDDS (filled symbols) and of an enantiomeric mixture of EDDS (open symbols). In the latter case, the [R,R] and [S,S] isomer could not be distinguished analytically, however, experiments with pure isomers showed that only [S,S]- EDDS was degradable.

Cell-free extracts of [S,S]-EDDS-grown cells and the purified EDDS-lyase catalyzed the degradation of [S,S]-EDDS as well as that of [R,S]-EDDS. But neither in the presence of cell-free extract nor of EDDS-lyase was a significant consumption of [R,R]-EDDS was observed over a time period of 5 hours. The degradability of the different EDDS isomers is summarized in Figure 4 showing the transformation of the enantiomeric mixture, of pure [R,R]-EDDS, and of [S,S]-EDDS in the presence of EDDS-lyase.

Discussion

The bacterial strain DSM 9103 originally isolated in our laboratory using EDTA as sole source of carbon, nitrogen, and energy (Witschel et al. 1997) is also capable to grow with [S,S]-EDDS, a structural isomers of EDTA. No growth, however, was observed with the R,R-enantiomer of EDDS. Moreover, whole cells were able to utilize the R,S-isomer. This is in accordance with the results of Schowanek and coworkers (1997), who tested the biodegradability of the EDDS isomers in both Sturm and Batch Activated Sludge Tests. They found that [S,S]-EDDS was readily biodegradable, whereas removal of [R,R]-EDDS was negligible. In these tests, [R,S]-EDDS was transformed but led to the formation of a recalcitrant intermediate identified as AEAA, most likely the D-isomer. [S,S]-EDDS seems to be the only naturally occuring stereo-isomer (Nishikiori et

al. 1984), whereas one must assume that [R,R]-EDDS and [R,S]-EDDS are not present in the environment yet. Therefore, it is likely that also a stereoselective degradation pathways for EDDS did evolve in microorganisms. Nevertheless, it should be pointed out that even for chiral compounds that are released as racemic mixture into nature, exclusive microbial degradation of only one enantiomer was observed. A marine microbial community, for example, degraded only the R-enantiomer of the herbicide dichlorprop [2-(2,4-dichlorphenoxy)propionic acid], whilst the S-enantiomer remained unaffected (Ludwig et al. 1992). In the case of the structurally related herbicide mecoprop [2-(2-methyl-4-chlorophenoxy)propionic acid], a bacterial consortium consisting of *Alcaligenes denitrificans, Pseudomonas glycinea,* and *Pseudomonas marginalis* was described to grow exclusively on [R]-mecoprop and not on [S-]-mecoprop (Tett et al. 1994). Zipper and coworkers (1996), again, isolated a bacterial strain unable to utilize both enantiomers of mecoprop but in an enantioselective manner with two enzyme activities each of them specific for the degradation of one enantiomer (Nickel et al. 1997). Moreover, it was reported that *Rhodococcus rhodochrous* can grow with the R-enantiomer of 3-phenylbutyric acid, an intermediate of linear alkylbenzene degradation, but not on the S-enantiomer (Simoni et al. 1996). All this information underlines the importance to treat enantiomers separately when the environmental fate of chiral compounds has to be evaluated. The results obtained for the degradability of the different EDDS isomers clearly indicate that only [S,S]-EDDS should be used for industrial applications.

The first step of the pathway for EDDS degradation was elucidated in DSM 9103. The product pattern of [S,S]-EDDS degradation in cell-free extracts of DSM 9103 was similar to those found for extracts of *C. asaccharovorans* and strain E1. This suggests that in all three strains the same type of enzymatic reaction led to the first attack of [S,S]-EDDS. In preliminary experiments, it was shown that the EDDS-transforming enzyme is inducible since cell-extracts from DSM 9103 grown with EDTA or fumarate or extracts from *C. asaccharovorans* grown with NTA exhibited no EDDS-degrading activity at all. In this context, it is noteworthy that [S,S]-EDDS triggered the induction of the EDTA-oxidizing system in DSM 9103 (Witschel et al. 1997), but that, in contrast, EDTA-grown cells did not show EDDS-degrading activity.

Moreover, the enzyme involved in the EDDS-transformation was purified and characterized from DSM 9103. The enzyme catalyzed the non-hydrolytic splitting of the C-N-bond between a succinyl-residue of EDDS and the ethylenediamine part. The products of this reaction were fumarate and AEAA (Figure 2). The enzyme catalyzed also the back-reaction, i.e. an addition of AEAA to the double bond of fumarate leading to the formation of EDDS. Although AEAA could not be determined analytically, the formation of EDDS from AEAA and fumarate proves that AEAA is really a product of the splitting reaction. All this indicates that the EDDS-degrading enzyme belongs to the group of carbon-nitrogen lyases, more precisely to the subgroup of amine lyases (EC 4.3.3), because a primary amine is formed during the reaction. Therefore, it is proposed that the systematic name [S,S]/[R,S]-ethylenediaminedisuccinate N-(2-aminoethyl) aspartate lyase is assigned to this enzyme. EDDS-lyase did not catalyze the removal of the second succinyl residue because no fumarate was formed when AEAA was added to the enzyme assay as the only substrate. The fact that no fumarate formation was detected for AEAA in cell-free extracts also indicates that the further metabolism of AEAA is based on another type of reaction. Moreover, when testing for the back-reaction, ethylenediamine instead of AEAA was not accepted as a substrate by the EDDS-lyase. This again underlines the hypothesis that AEAA is not further metabolized by the lyase. One can speculate that, catalyzed by a dehydrogenase or a monooxygenase, the C-N bond between the succinyl residue and the ethylenediamine part of the molecule is split, or that an aspartyl residue is removed by cleavage of the C-N bond within the ethylenediamine part of AEAA.

From the different stereo-isomers of EDDS, EDDS-lyase accepted [S,S]- and [R,S]-EDDS. This indicates that only the S-configuration of the C-atom involved in the C-N-bond to be split can be attacked by the enzyme. Consequently, one must assume that out of fumarate and a racemic mixture of AEAA, [S,S]- and [R,S]-EDDS but no [R,R]-EDDS were formed.

EDDS-lyase accepted free [S,S]-EDDS as well as EDDS-complexes with earth alkaline metals and MnEDDS as substrate. All readily degradable complexes have stability constants lower than 8 and MnEDDS, having the highest stability constant ($LogK_{Mn}$ = 8.9) among the degradable complexes, was transformed only at a very low rate. All complexes exhibiting stability constants higher than 10 were not degraded at all (stability constants were taken from

Martell & Smith 1974). This suggests a connection between stability constant and degradability of a complex, which is opposite to the results obtained for the degradation of EDTA or NTA catalyzed by EDTA- or NTA-oxidizing system, where no such relationship between the stability constant and the degradability of the different metal complexes was found (Witschel et al. 1997; Xun et al., 1996). Thus, presently no general rule concerning the enzymatic degradability of metal complexes of aminopolycarboxylic acids as function of their stability constant can be deduced. An explanation for the close link between stability constant of metal-[S,S]-EDDS-complexes and their enzymatic degradability could be that during the process of substrate binding the metal ligand of the [S,S]-EDDS molecule has to be exchanged or removed. This step in the reaction might become inhibited in the case of metal-[S,S]-EDDS-complexes with high stability constants.

It is interesting that, in the presence of metal ions, the equilibrium between [S,S]-EDDS and the two products of the enzymatic degradation, fumarate and AEAA, was shifted towards [S,S]-EDDS. Under these conditions, there are three other equilibria which play a role: first of all the equilibrium between uncomplexed and complexed [S,S]-EDDS as well as the equilibria between the uncomplexed products fumarate and AEAA and their complexed forms. As far as we are aware, the stability constants for metal-AEAA complexes are not known, but it seems reasonable that they are lower than those of [S,S]-EDDS and higher than those of fumarate. Since out of the three compounds, [S,S]-EDDS is the ligand exhibiting the highest stability constants, more of the educt [S,S]-EDDS than of the two products is removed from the equilibrium of the lyase catalyzed reaction in the presence of metal ions. Hence, the equilibrium will be shifted towards [S,S]-EDDS.

The metal binding constants for [R,S]-EDDS are most likely lower than those of [S,S]-EDDS due to the fact that in the [R,S] isomer probably one of the carboxylate groups is directed away from the metal as it was shown for the Co[R,S]-EDDS complex anion by analysis of the crystal structure (Pavelcik et al. 1980). This would explain the observation that in the back-reaction less [R,S]-EDDS and more [R,R]/[S,S]-EDDS was formed in the presence of metals.

In the proposed pathway for the synthesis of [S,S]-EDDS by *A. orientalis*, AEAA is postulated to be an intermediate (Cebulla 1995). It was suggested that AEAA reacts with oxaloacetate by formation of a Schiff's base which is then reduced to [S,S]-EDDS. However, there is no evidence yet that this reaction of AEAA with oxaloacetate takes place. It would also be possible that a lyase was involved in the synthesis of [S,S]-EDDS starting from the postulated intermediate AEAA and fumarate. In fact, the reversible nature of the EDDS-lyase catalyzed reaction fosters the speculation that this enzyme might also play a role in the synthesis of EDDS. Therefore, it would be interesting to test whether EDDS-lyase activity is present in cell-free extracts of *A. orientalis*. This could give an indication whether or not the EDDS-lyase is responsible for both [S,S]-EDDS degradation and [S,S]-EDDS synthesis.

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