Crystal Structure of the Ectoine Hydroxylase, a Snapshot of the Active Site* 

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Background: 5-Hydroxyectoine is a compatible solute synthesized by the ectoine hydroxylase (EctD), a non-heme containing iron(II) and 2-oxoglutarate-dependent dioxygenase.

Results: The structure of EctD was solved in different forms.

Conclusion: The architecture of the catalytic core of EctD was revealed.

Significance: The crystal structure increases our understanding of the structural-functional relationship in an evolutionary conserved group of enzymes.

Ectoine and its derivative 5-hydroxyectoine are compatible solutes that are widely synthesized by bacteria to cope physiologically with osmotic stress. They also serve as chemical chaperones and maintain the functionality of macromolecules. 5-Hydroxyectoine is produced from ectoine through a stereo-specific hydroxylation, an enzymatic reaction catalyzed by the ectoine hydroxylase (EctD). The EctD protein is a member of the non-heme-containing iron(II) and 2-oxoglutarate-dependent dioxygenase superfamily and is evolutionarily well conserved. We studied the ectoine hydroxylase from the cold-adapted marine ultra-microbacterium Sphingopyxis alaskensis (Sa) and found that the purified SaEctD protein is a homodimer in solution. We determined the SaEctD crystal structure in its apo-form, complexed with the iron catalyst, and in a form that contained iron, the co-substrate 2-oxoglutarate, and the reaction product of EctD, 5-hydroxyectoine. The iron and 2-oxoglutarate ligands are bound within the EctD active site in a fashion similar to that found in other members of the dioxygenase superfamily. 5-Hydroxyectoine, however, is coordinated by EctD in a manner different from that found in high affinity solute receptor proteins, operating in conjunction with microbial import systems for ectoines. Our crystallographic analysis provides a detailed view into the active site of the ectoine hydroxylase and exposes an intricate network of interactions between the enzyme and its ligands that collectively ensure the hydroxylation of the ectoine substrate in a position- and stereo-specific manner.

Cells of all three kingdoms of life exploit compatible solutes as protectants against water loss that ensues upon their exposure to high osmolarity surroundings (1, 2). These organic compounds can be accumulated to exceedingly high intracellular concentrations without exerting negative side effects on the biochemistry of the cell. They thus serve as physiologically compliant water-attracting osmolytes to maintain an adequate level of cellular hydration (3). The physicochemical attributes of compatible solutes that make their physiological function(s) possible largely stem from their negative interactions with the protein backbone that in turn leads to their preferential exclusion from protein surfaces (4). As a consequence of the resulting imbalance of the distribution of compatible solutes in the cell water, thermodynamics favor the native conformation of proteins and the functionality of other macromolecules under otherwise function-disrupting conditions (5, 6). The term "chemical chaperone" has been coined in the literature to reflect these beneficial traits (7).

Ectoine ((4S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) (Fig. 1) (8) is a well recognized compatible solute and microbial stress protectant (9, 10). Its function-preserving (10–15) and anti-inflammatory properties fostered substantial interest for its industrial scale production (9, 16) and exploitation in various biotechnological applications, in skin-care products (9, 10, 16–18), and in potential medical uses (19–21). Ectoine is synthesized widely by members of bacteria (10, 16, 22) as an adaptive response to osmotic stress (16, 23–29) and growth-restricting extremes in high and low temperatures (25, 30). Synthesis of ectoine relies on the supply of the precursor l-aspartate-β-semialdehyde and is carried out through the enzymatic

* This work was supported in part by Deutsche Forschungsgemeinschaft (Grant SFB 987, the LOEWE program of the State of Hessen (via the Center for Synthetic Microbiology, Marburg), the Max-Planck Institute for Terrestrial Microbiology (Marburg) through the Emeritus Group of R. K. Thauer, a contribution by the Fonds der Chemischen Industrie, by the Heinrich-Heine-University Düsseldorf and its Institute of Biochemistry, and by the Heine-University Düsseldorf and its Institute of Biochemistry, Universitätsstr. 1, D-40225 Düsseldorf, Germany. Tel.: 49-211-8112647; Fax: 49-211-8115310; E-mail: Sander.Smits@hhu.de.

† Recipient of a Ph.D. fellowship from the International Max Planck Research School for Environmental, Cellular, and Molecular Microbiology, Marburg, Germany.

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§ **3 This work was supported in part by Deutsche Forschungsgemeinschaft (Grant SFB 987, the LOEWE program of the State of Hessen (via the Center for Synthetic Microbiology, Marburg), the Max-Planck Institute for Terrestrial Microbiology (Marburg) through the Emeritus Group of R. K. Thauer, a contribution by the Fonds der Chemischen Industrie, by the Heinrich-Heine-University Düsseldorf and its Institute of Biochemistry, and by the Heine-University Düsseldorf and its Institute of Biochemistry, Universitätsstr. 1, D-40225 Düsseldorf, Germany. Tel.: 49-211-8112647; Fax: 49-211-8115310; E-mail: Sander.Smits@hhu.de.

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activities of L-2,4-diaminobutyrate transaminase (EctB; EC 2.6.1.76), 2,4-diaminobutyrate acetyltransferase (EctA; EC 2.3.1.178), and ectoine synthase (EctC; EC 4.2.1.108) (22, 31, 32).

A substantial group of ectoine producers (22) also synthesize a derivative in which a hydroxy group is attached to the pro-5 hydrogen at position C5 in the tetrahydropyrimidine ring of ectoine in a stereo-specific manner, thereby yielding 5-hydroxyectoine ((4S,5S)-5-hydroxy-2-methyl-1,4,5,6-tetrahydro-4-carboxylic acid (Fig. 1) (33), a reaction carried out by the ectoine hydroxylase (EctD; EC 1.14.11.141) (27, 30, 34). Despite their closely related chemical structures, 5-hydroxyectoine is often superior to its precursor molecule ectoine in protecting microorganisms against environmentally imposed stresses (26, 30) and in preserving the functionality of macromolecules and cells (11–15, 35, 36). This makes a better understanding of the ectoine hydroxylase desirable, both in terms of basic science and for its practical use in biotechnological processes.

The ectoine hydroxylase is a member of the non-heme-containing iron(II) and 2-oxoglutarate-dependent dioxygenase superfamily, a group of versatile biocatalysts that not only carry out hydroxylation reactions but also dimethylation, desaturations, cyclizations, ring expansions, epimerizations, and halogenations (37–41). Although the type of substrates used by these dioxygenases varies considerably, common enzyme reaction mechanisms are observed. Most members of this superfamily couple the two-electron oxidation of their substrates with the reaction of oxygen and 2-oxoglutarate (38, 39, 42). The ectoine hydroxylase adheres to this general reaction scheme (27, 43).

Despite their varied enzyme activities, non-heme-containing iron(II) and 2-oxoglutarate-dependent dioxygenases are structurally closely related and typically consist of a double-stranded β-helix (DSBH)5 core, the so-called jelly roll or cupin fold (37–40). Crystallographic analysis of the ectoine hydroxylase from the salt-tolerant moderate halophile Virgibacillus salensis (Vs) in its iron-bound (44) and iron-free (22) forms revealed that the DSBH in the VsEctD protein is formed by four-stranded antiparallel β-sheets arranged in form of a β-sandwich; it is decorated with and stabilized by a number of α-helices (22, 44). However, both available crystal structures of the VsEctD protein lack the co-substrate 2-oxoglutarate and the substrate ectoine, and consequently, our understanding of the structure and architecture of the active site of this enzyme is incomplete.

Recently, we have biochemically characterized six ectoine hydroxylases from microorganisms that are able to colonize habitats with extremes in salinity, growth temperature, and pH values (22). These six enzymes possess kinetic parameters similar to those of the previously studied ectoine hydroxylases from V. salensis and Streptomyces coelicolor (26, 27), but differences in their ability to withstand the inhibiting effects of high salt concentrations and high temperature were noted for some of them (22). These enzymes might thus be better suited than the V. salensis EctD protein to obtain a crystal structure containing all ligands.

Here, we report the crystal structures of the ectoine hydroxylase from the cold-adapted marine ultra-microbacterium Sphingopyxis alaskensis (45), SaEctD (22, 46), in its apo-form, in complex with iron and in a form that contains the iron catalyst, the co-substrate 2-oxoglutarate and the product of the EctD-catalyzed enzyme reaction, 5-hydroxyectoine. Combined with previous molecular dynamics simulations (44), the crystallographic analysis presented here and our site-directed mutagenesis experiments revealed the architecture of the active site of this evolutionarily well conserved group of enzyme.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Ectoine and 5-hydroxyectoine were kindly provided by Dr. Thomas Lentzen and Dr. Irina Bagyan (bitop AG, Witten, Germany). 2-Oxoglutarate (disodium salt) was purchased from Sigma. Anhydrotetracycline hydrochloride, deshioibiotin, and Strep-Tactin Superflow chromatography material were obtained from IBA GmbH (Göttingen, Germany).

**Bacterial Strains, Media, and Growth**—Escherichia coli strain DH5α (Invitrogen) was used for the propagation of the ectD+ plasmid pMP40 (22, 46). It was grown at 37°C in Luria-Bertani (LB) liquid medium or on LB agar plates containing ampicillin (100 µg ml⁻¹) to select for the presence of the plasmid. Heterologous overproduction of the S. alaskensis EctD protein was carried out in the E. coli B strain BL21 (pMP40) in minimal medium A (47) containing 0.5% (w/v) glucose as carbon source, 0.5% (w/v) casamino acids, 1 mM MgSO₄, and 3 mM thiamine.

**Site-directed Mutagenesis of the Sa-ectD Gene**—To assess the contribution of amino acids predicted by structural analysis to be involved in the binding of the 5-hydroxyectoine molecule, or predicted to contribute to interactions between the SaEctD monomers, we performed site-directed mutagenesis. For these experiments, we used the QuikChange Lightning mutagenesis kit (Stratagene, La Jolla, CA) and custom-synthesized mutagenic DNA primers; the DNA primers had a typical length of 25 nucleotides and were purchased from Biomers (Ulm, Germany). To ensure the presence of the desired mutation(s) and the absence of unwanted alterations within the mutagenized ectD gene, the DNA sequence of the entire coding region was determined (Eurofins MWG GmbH, Ebersberg, Germany) for each ectD mutant allele. DNA of plasmid pMP40 carrying the Sa-ectD wild-type gene was used as the template for the site-directed mutagenesis experiments. The following Sa-ectD gene variants were isolated: pWN20 (Gln-127 to Ala (CAG to GCG)), pWN21 (Arg-139 to Ala (AGG to GCG)), pWN22 (Glu-140 to Ala (GAG to GCG)), pWN23 (Thr-149 to Ala (ACC to GGC)), and pWN24 (Asp-151 to Asn (GAT to GTA)).

**Crystal Structure of the Ectoine Hydroxylase**

![Chemical structures of ectoine and 5-hydroxyectoine.](image-url)

FIGURE 1. Chemical structures of ectoine and 5-hydroxyectoine.
Crystal Structure of the Ectoine Hydroxylase

GCCCCCCCG GC, pWN24 (Trp-150 to Ala (TGG to GCG)), pWN25 (Arg-280 to Ala (CGC to GCC)), and the double mutant pWN26 (Arg-139 to Ala (AGG to GCG) and Glu-140 to Ala (GAG to GCG)). These SaEctD variants were overproduced in the E. coli B strain BL21 and purified by affinity chromatography. They were functionally assessed by the previously described ectoine hydroxylase enzyme assay (22, 46).

Overproduction and Purification of the Recombinant EctD Protein—PmpMP40 is a derivative of the expression vector pASK-IBA3 (IBA, Göttingen, Germany) in which the transcription of the ectD gene (accession number YP_617990) from S. alaskensis (45) is positioned under the control of the TetR-responsive and anhydrotetracycline hydrochloride-inducible tet promoter carried by the backbone of the pASK-IBA3 expression vector (43, 46). In this plasmid, a nine-amino acid Strep-tag-II affinity peptide encoded by a short synthetic DNA sequence is fused to the 3’ end of the ectD-coding region to allow the purification of the encoded SaEctD-Tag-II protein by affinity chromatography using Strep-Tactin Superflow material. Overproduction and purification of this recombinant protein for crystallization experiments was carried out as described (46). 200–300 mg of SaEctD-Strep-II protein/liter of culture was routinely obtained, and the protein was concentrated with Vivaspin 6 columns (Sartorius Stedim Biotech GmbH, Göttingen, Germany) to about 10 mg/ml of culture. Overproduction and purification of this recombinant protein by affinity chromatography using Strep-Tactin Superflow allow the purification of the encoded sequence is fused to the 3’end of the expression vector (43, 46). In this plasmid, a nine-amino acid Strep-tag-II affinity peptide encoded by a short synthetic DNA sequence is fused to the 3’end of the ectD-coding region to allow the purification of the encoded SaEctD-Strep-tag-II protein by affinity chromatography using Strep-Tactin Superflow material. Overproduction and purification of this recombinant protein for crystallization experiments was carried out as described (46). 200–300 mg of SaEctD-Strep-tag-II protein/liter of culture was routinely obtained, and the protein was concentrated with Vivaspin 6 columns (Sartorius Stedim Biotech GmbH, Göttingen, Germany) to about 10 mg/ml (in 20 mM TES buffer, pH 7.5, 80 mM NaCl) before it was used for crystallization trials. The purity of the obtained SaEctD-Strep-tag-II protein preparations was assessed on 12% SDS-polyacrylamide gels, and their iron content was determined with the aid of a colorimetric assay (27, 48).

Ectoine Hydroxylase Enzyme Activity Assay—Ectoine hydroxylase enzyme activity of the SaEctD-Strep-tag-II protein was determined under previously described buffer and assay conditions (26, 27, 46). Specifically, we monitored the EctD-mediated conversion of ectoine into 5-hydroxyectoine in a 30-μl reaction volume that contained 6 mM ectoine, 10 mM 2-oxoglutarate, 1 mM FeSO₄, 20 mM TES, pH 8.0, and 100 mM KCl. 15 μg of either the EctD wild-type protein or the particular SaEctD mutant protein under study was added to the reaction mixture. Each assay was run for 10 min at 40 °C under vigorous aeration. The enzyme reaction was stopped by adding 30 μl of acetonitrile (100%) to the reaction vessel. The substrate ectoine and the reaction product 5-hydroxyectoine were analytically separated from each other on a GROM-SIL Amino-1PR column (125 × 4 mm with a particle size of 3 μm); it was purchased from GROM (Rottenburg-Hailfingen, Germany). The conversion of ectoine to 5-hydroxyectoine by the purified SaEctD-Strep-tag-II protein was monitored with a UV-visible detector integrated into a FPLC system (SYKAM, Fürstenfeldbruck, Germany) by measuring the absorbance of these solutes at a wavelength of 210 nm (22, 24, 27). The amount of ectoine remaining in the enzyme assay and the amount of 5-hydroxyectoine formed by the wild-type and mutant EctD proteins were quantified using the ChromStar 7.0 software package (SYKAM, Fürstenfeldbruck, Germany). The data shown for each mutant were derived from two independent SaEctD preparations, and each SaEctD protein solution was assayed three times for its enzyme activity.

Determination of the Oligomeric State of Purified SaEctD and Mutant Derivatives by Conventional Size-exclusion Chromatography and HPLC-MALS—To determine the oligomeric state of the SaEctD-Strep-tag-II protein in solution, we used conventional size-exclusion chromatography and high performance liquid chromatography coupled to multangle light scattering detection (HPLC-MALS). Size-exclusion chromatography was performed with a HiLoad 16/600 Superdex 2000 pg column (GE Healthcare) equilibrated and run in a 20 mM TES buffer, pH 7.5, containing 150 mM NaCl under conditions described previously (22). The size-exclusion column was standardized with a gel filtration markers kit (Sigma). For HPLC-MALS analysis, a Bio SEC-5 HPLC column (Agilent Technologies Deutschland GmbH, Böblingen, Germany) with a pore size of 300 Å was equilibrated with 20 mM TES, pH 7.5, 80 mM NaCl for HPLC using a system from Agilent Technologies connected to a triple-angle light-scattering detector (miniDAWN TREOS, Wyatt Technology Europe GmbH, Dernbach, Germany) followed by a differential refractive index detector (OPTILab T-rEX, Wyatt Technology). Typically, 100 μl of purified SaEctD (2.0 mg ml⁻¹) was loaded onto the Bio SEC-5 HPLC column, and the obtained data were analyzed with the ASTRA software package (Wyatt Technology).

Crystallization of the SaEctD Protein—For apo-SaEctD, the crystallization trials were performed using the sitting-drop vapor diffusion method at 20 °C. 1.5 μl of the homogeneous protein solution of SaEctD-Strep-tag-II (10 mg ml⁻¹ in 20 mM TES, pH 7.5, 80 mM NaCl) was mixed with 1.5 μl reservoir solution containing 100 mM MES, pH 6.0, 200 mM calcium acetate, 30% (w/v) PEG 400, and 1.5 mM n-dodecyl-N,N-dimethylglycine and equilibrated over the 300 μl reservoir solution. Crystals grew within 6–12 days to their final size of around 30 × 30 × 50 μm³. For Fe-SaEctD-Strep-tag-II in complex with Fe²⁺, the SaEctD-Strep-tag-II enzyme in complex with its iron catalylist was crystallized as described above for apo-SaEctD-Strep-tag-II protein except that the protein solution was pre-mixed with 100 mM Fe(II)Cl₂ to a final concentration of 4 μM and incubated on ice for 10–15 min. In addition, the solution contained 3.5 μM n-dodecyl-N,N-dimethylglycine. Crystals grew within 6–12 days at 20 °C to their final size of around 40 × 40 × 180 μm³. For Fe-SaEctD-Strep-tag-II in complex with 2-oxoglutarate and 5-hydroxyectoine, the SaEctD-Strep-tag-II protein was mixed with Fe(II)Cl₂ as described above, and 2-oxoglutarate was subsequently added to the protein solution to a final concentration of 40 mM. After 30 min of incubation on ice, 5-hydroxyectoine was added to the solution to a final concentration of 40 mM. This mixture was then incubated for 1 h on ice before crystallization trials were conducted. SaEctD crystals were grown by mixing 1.5 μl of protein solution with 1.5 μl of reservoir containing 100 mM MES, pH 6.0, 200 mM calcium acetate, 30% (w/v) PEG 400, and 25 mM n-octyl-β-D-glycoside; they grew within 6–12 days to their final size of around 40 × 40 × 70 μm³. All crystals were cryoprotected by carefully adding 1 μl of 100% glycerol to the crystallization drop before the crystals were frozen in liquid nitrogen.

Data Processing and Structure Determination—Data sets were collected from a single crystal obtained from the various crystallization trials (apo-SaEctD, Fe-SaEctD, and Fe-SaEctD/
2-oxoglutarate/5-hydroxyectoine) at the ESRF beamline ID23eh2 (Grenoble, France) at 100 K. These data sets were processed using the XDS package (49) and scaled with XSCALE (50). Initial phases were obtained by molecular replacement using the program Phaser (51) with the crystal structure of the V. salinis ectD protein (PDB code 3EMR) as the template (44). Model building and refinement were performed using COOT (52) and REFMAC5 (53). Data refinement statistics and model content are summarized in Table 1. The structures were deposited at the Protein Data Bank (Brookhaven, NY) under the following accession codes: 4MHX for the apo-SaEctD protein, 4MHI for the Fe-SaEctD protein, and 4Q5O for Fe-SaEctD/2-oxoglutarate/5-hydroxyectoine crystal structure.

Database Searches for EctD-related Proteins—We used the amino acid sequence (accession number YP_617990) from S. alaskensis (45) to search microbial genomes for EctD-related proteins in the databases of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and of the United States Department of Energy Joint Genome Institute (jgi.doe.gov). Amino acid sequences of EctD-type proteins were aligned as described previously (22, 43).

**Figure Preparation of Crystal Structures**—Figures of the crystal structure of the SaEctD protein were prepared using the PyMOL software package and Chimera.

**RESULTS**

**Biochemical Properties of the Ectoine Hydroxylase from S. alaskensis and its Oligomeric State in Solution**—The ectoine hydroxylase studied here originates from S. alaskensis, a microorganism that is well adapted to a life in chilly ocean water systems (45). Despite this permanently cold habitat of the producer microorganism, the SaEctD enzyme itself is thermostable (22). The kinetic properties of the SaEctD protein (with $K_m$ values of 9.8 ± 0.5 and 2.7 ± 0.3 mM for the substrate ectoine and the co-substrate 2-oxoglutarate, respectively, and a catalytic efficiency ($k_{cat}/K_m$) of 0.12 mm$^{-1}$ s$^{-1}$) resemble those of other biochemically studied ectoine hydroxylases (22, 26, 27).

We used for our studies a recombinant SaEctD-Strep-tag-II protein that carries a nine-amino acid tag at its carboxy terminus to allow affinity purification on Strep-Tactin Superflow material (22, 46). To determine the oligomeric state and to validate the monodispersity of the EctD protein in solution, we carried out a HPLC-MALS analysis. The normalized elution profiles from the UV, refractive index, and light scattering detectors revealed one symmetric peak indicating that the protein solution was homogeneous and monodisperse. After determining protein concentration by the refractive index, a molecular mass of 70.73 ± 1.1 kDa was obtained for the SaEctD-Strep-tag-II protein (Fig. 2). This molecular mass corresponds very well to the theoretical molecular mass of a dimer of the recombinant protein (calculated molecular mass of the monomer, including the Strep-tag-II affinity peptide: 35.29 kDa) (46). We therefore conclude that the ectoine hydroxylase from S. alaskensis is a dimer in solution, a conclusion that is in agreement with the suggested quaternary structures of six other EctD proteins that have been assessed by conventional size exclusion chromatography (22).

**Crystallography of the SaEctD-Strep-tag-II Protein**—By slightly varying the previously used overproduction conditions for the SaEctD-Strep-tag-II protein in E. coli (22), we were able to increase the amounts of the recovered SaEctD-Strep-tag-II protein/liter of culture about 10-fold to 200–300 mg (46). However, although the modified overproduction procedure aided the provision of large amounts of protein for crystallization trials, this also reduced the iron content (44) of the SaEctD-Strep-tag-II protein preparation to about 0.13 mol of iron/mol of protein and thereby caused a strong decrease in ectoine hydroxylase activity. By adding FeSO$_4$ or FeCl$_2$ to the purified protein solution, the ectoine hydroxylase activity could be restored, and this allowed us to obtain crystals of the SaEctD-Strep-tag-II protein in its apo- and iron-bound forms. The crystals of the apo-SaEctD and Fe-SaEctD proteins diffracted to a resolution of 2.1 and 2.6 Å, respectively.

Co-crystallization experiments of SaEctD with its substrate ectoine were not successful, despite that a large number of well diffracting crystals were obtained; however, none of these contained the ectoine ligand as revealed by x-ray analysis. The co-crystallization of a protein with its enzymatic reaction product has been successfully used to obtain informative crystal structures (54). When applied to the SaEctD protein, it yielded crystals with good diffraction properties, but none of them showed any electron density for 5-hydroxyectoine. We then turned to an approach in which we added iron, 2-oxoglutarate, and 5-hydroxyectoine in a stepwise manner to the SaEctD-Strep-tag-II protein in which we incubated the protein solution containing a high concentration of the ligands for at least 1.5 h on ice before it was used for crystallization trials. In this way, we obtained crystals that diffracted to a resolution of 2.56 Å and that contained the iron ligand, the co-substrate 2-oxoglutarate, and the EctD-enzyme reaction product 5-hydroxyectoine.

All three crystal structures of the SaEctD protein were solved by molecular replacement using the crystal structure of the V. salinis (VsEctD) ectoine hydroxylase (PDB code 3EMR) (44) as the search model. A summary of the data collection statistics, refinement details, and model content is given in Table 1.

**Overall Fold of the SaEctD Protein**—The amino acid sequences of the VsEctD and SaEctD possess an amino acid
sequence identity of 51% (for an alignment see Fig. 3). An overall comparison of the three newly determined SaEctD crystal structures with that of the previously determined structure of VsEctD revealed a high degree of identity with an r.m.s.d. that ranges from 1.3 to 1.6 Å over 279 C-α atoms. An even lower r.m.s.d. of 0.5–0.8 Å was found when the three different SaEctD structures were compared with each other. Hence, neither the binding of the catalytically critical iron ligand nor of the substrate 2-oxoglutarate and 5-hydroxyectoine triggered a strong change in the overall conformation of the EctD protein.

Because the monomers of all three SaEctD structures are nearly identical in overall shape, we describe in the following section only the overall fold for the apo-SaEctD protein. The SaEctD crystal structure consists of a double-stranded β-helix core surrounded and stabilized by a number of α-helices (Fig. 4A). This core, also known as the jelly roll or cupin fold (38, 39), is formed by two four-stranded anti-parallel β-sheets that are arranged in the form of a β-sandwich (Fig. 4A). This type of architecture has previously been observed not only for the VsEctD structure (22, 44) but also for many other non-heme-containing iron(II) and 2-oxoglutarate-dependent dioxygenases (38–40). Examples are the EctD-related structures of the human phytanoyl-CoA hydroxylases PhyH (PDB code 2A1X; r.m.s.d. of 2.6 Å over 252 C-α atoms when compared with the SaEctD structure) (55) and PhyHD1A (PDB code 2OPW r.m.s.d. of 2.2 Å over 286 C-α atoms) (56) and the halogenases SyrB2 from Pseudomonas syringae (PDB code 2FCV; r.m.s.d. of 2.9 Å over 299 C-α atoms) (57), CytC3 from a Streptomyces soil isolate (PDB code 3GA; r.m.s.d. of 3.0 Å over 204 C-α atoms) (58), and CurA from the cyanobacterium Lyngbya majuscula (PDB code 3NNM; r.m.s.d. of 3.3 Å over 222 C-α atoms) (59). This structural comparison shows that the core of the EctD protein, its cupin-type fold, is very similar to these proteins; however, the rest of EctD differs from the structurally compared proteins and thereby yields higher r.m.s.d. values.

In each of the three SaEctD crystal structures, a large loop (amino acids 191–210) is disordered and hence not visible in the electron density map; the corresponding region was therefore not included in the SaEctD models. By amino acid sequence alignment (Fig. 3), this loop corresponds to residues 195–211 in the VsEctD ectoine hydroxylase, and it is also disordered in both crystal structures of this protein (22, 44). Notably, in the ligand-free crystal structures of the phytanoyl-CoA hydroxylases PhyH (55), the asparagine hydroxylase AsnO from S. coelicolor (60), the l-arginine oxygenase VioC from Streptomyces vinaceus (61), and the taurine dioxygenases from E. coli and Pseudomonas putida (62), a similar loop is disordered as well and became only visible in crystal structures with bound substrates. It is thought that this mobile loop functions as a lid that shields the enzyme reaction chamber during catalysis from the solvent.

**Dimer Interface of the SaEctD Protein**—The three crystal structures of the ectoine hydroxylase from S. alaskensis displayed a different crystal packing (as observed by the different symmetry of the crystals) and protein composition in the asymmetric unit (ASU) of the crystals. In apo-SaEctD, the asymmetric unit contains a monomer, whereas the ASUs of Fe-SaEctD and Fe-SaEctD/2-oxoglutarate/5-hydroxyectoine both include dimers. The monomers in the latter dimer are however differently oriented (Fig. 5). Because the SaEctD protein is a dimer in solution (see above and Fig. 2), we inspected the crystal packing and analyzed the monomer/monomer interactions to elucidate...
which dimer in the crystals might represent the physiologically relevant dimer (Fig. 5). The monomers in the ASUs of apo-SaEctD form also a dimer assembly through crystal symmetry, similar to that found in the SaEctD/2-oxoglutarate/5-hydroxyectoine crystal structure. The monomers in the Fe-SaEctD dimer are slightly shifted toward each other when
one compares them to the $SaEctD/2$-oxoglutarate/5-hydroxyectoine dimer. However, this difference in the Fe-$SaEctD$ structure is likely due to the extra $n$-dodecyl-$N,N$-dimethylglycine molecule that was present in the crystallization solution and that is bound in between the two monomers. This leads to a different crystal packing in the ASU. In contrast, when looking at the Fe-$SaEctD$ dimer composed of the symmetry-related monomers (Fig. 5B, right panel), we found the same arrangement in the ASU of the $SaEctD/2$-oxoglutarate/5-hydroxyectoine crystal (Fig. 5C). The only other member of the EctD superfamily that has been so far crystallized is the $VsEctD$ protein in forms that either possess or lack the iron catalyst (22, 44). In these structures, the ASU contains a monomer, although gel filtration analysis showed that the $VsEctD$ protein is a dimer in solution (22). We re-analyzed the crystal packing of the $VsEctD$ protein and found a similar dimer as observed in the $SaEctD/$
2-oxoglutarate/5-hydroxyectoine crystal structure presented here (Fig. 5D).

To ensure that the dimer observed in the SaEctD/2-oxoglutarate/5-hydroxyectoine crystal structure is also a dimer in solution, we studied its quaternary composition under the very same buffer and substrate conditions that were used in the co-crystallization experiments by analyzing these samples using size-exclusion chromatography-MALS. The molecular mass determined for the SaEctD-Strep-tag-II protein by this experiment was 75.02 ± 1.8 kDa, which corresponds to a dimeric state of the protein (calculated theoretical mass of the iron, 2-oxoglutarate, and 5-hydroxyectoine bound dimer, 71.3 kDa). We then analyzed the dimer interface using the PDBePISA software (63). We found 10 potential monomer/monomer interaction regions (Table 2) and observed that the involved amino acids are all located in loop areas pointing from one monomer to the other monomer (Fig. 4B). However, inspection of the aforementioned loop regions implicated in forming the dimer interface revealed that their amino acid sequences are only moderately conserved, as judged by consulting a previous alignment of 433 EctD-type proteins (22, 43).

Residues Arg-139 and Glu-140 seemed to make most contributions to dimer formation (Table 2). To investigate their potential role for the quaternary structure and their possible corresponding influence on enzyme activity, we targeted these residues of SaEctD by site-directed mutagenesis. We replaced them individually by Ala residues and also constructed the respective double mutant. Each of these EctD variants was fully catalytically active (Table 3). Contrary to expectations, each of these EctD proteins still formed dimers in solution, as assessed by conventional size-exclusion chromatography. This is documented for the double mutant EctD protein (Arg-139/Ala and Glu-140/Ala) in Fig. 6.

TABLE 2
Monomer/monomer interactions in the SaEctD protein in complex with Fe-2-oxoglutarate and 5-hydroxyectoine
The amino acids involved in the dimer interface are listed, and the distance and type of interaction ([H], hydrogen bond; [S], salt bridge) are indicated.

| Monomer A   | Monomer B   | Distance (type) |
|-------------|-------------|-----------------|
| Glu-140 (OE1) | Arg-139 (NH2) | 2.6 Å (H)      |
| Glu-140 (OE1) | Arg-139 (NH1) | 3.7 Å (S)      |
| Glu-140 (OE1) | Arg-139 (NH2) | 3.7 Å (S)      |
| Glu-140 (OE1) | Arg-139 (NE) | 2.5 Å (S)      |
| His-174 (NE2) | Ile-226 (O)  | 3.3 Å (H)      |
| Glu-214 (OE2) | Lys-137 (NZ) | 3.6 Å (S)      |
| Gly-249 (O)  | Asn-248 (ND2) | 3.3 Å (H)     |
| Ile-251 (O)  | Trp-143 (NE1) | 3.1 Å (H)     |

TABLE 3
Conversion of ectoine into 5-hydroxyectoine by SaEctD mutants
The conversion of ectoine into 5-hydroxyectoine by the SaEctD protein was monitored in a reaction that contained 6 mM ectoine as the substrate, 10 mM 2-oxoglutarate as the co-substrate, and 15 µg of the EctD protein under study. The amount of 5-hydroxyectoine was measured after 10 min.

| EctD variant | 5-Hydroxyectoine | Proposed role in |
|--------------|------------------|-----------------|
| Wild type    | 4.93 ± 0.26      | Ectoine binding |
| Q127A        | 0.04 ± 0.03      | Ectoine binding |
| T149A        | 0.17 ± 0.05      | Ectoine binding |
| W150A        | 0.09 ± 0.06      | Ectoine binding |
| R280A        | 0.31 ± 0.14      | Ectoine binding |
| R139A        | 5.01 ± 0.15      | Dimerization    |
| E140A        | 4.93 ± 0.20      | Dimerization    |
| R139A/E140A  | 4.72 ± 0.31      | Dimerization    |

FIGURE 6. Size-exclusion chromatography of the SaEctD double mutant (R139A/Q140A). The purified mutant protein (5 µg) was loaded onto a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) equilibrated and run in a 20 mM TES buffer, pH 7.5, containing 150 mM NaCl. The column run was performed at 15 °C. The size-exclusion column was standardized with a gel filtration markers kit (Sigma). The elution profile of the mutant EctD protein is shown, and arrows indicate that of relevant marker proteins. The inset shows a plot of the molecular mass (given in Da) of marker proteins (black dots) and of the EctD (R139A/Q140A) variant (red dot) versus the elution volume (given in ml).
Architecture of the Iron-binding Site—Because the overproduction conditions perfected for the large scale isolation of the ectoine hydroxylase in E. coli (see above) foster the synthesis of iron-free recombinant SaEctD-Strep-tag-II protein, we were able to obtain SaEctD crystal structures that either possessed or lacked the iron catalyst (Fig. 7, A and B). This was accomplished by adding or leaving out Fe(II)Cl₂ in the crystallization trials.

The iron ligand is bound in the SaEctD protein via interaction with two histidine side chains, His-144 and His-245, and that of Asp-146 (Fig. 7B). These residues form a structurally well conserved HX(D/E) . . . H motif, the so-called 2-His-1-carboxylate facial triad, forming a type of mononuclear iron center found in many members of the dioxygenase superfamily (37–40, 64). The three iron-binding residues are fully conserved among a group of 433 aligned EctD-type proteins (22). Two of the iron-coordinating residues (His-144 and Asp-146) in the SaEctD protein are part of a 17-amino acid residue region that is strictly conserved in ectoine hydroxylases (22, 27, 44).

FIGURE 7. Crystal structures of the SaEctD-Strep-tag-II protein in its apo- and iron-bound forms and in complex with iron, 2-oxoglutarate, and 5-hydroxyectoine. Ribbon representation of the catalytic core of the iron-free (PDB code 4HMR) (A) and iron-bound (PDB code 4MHU) (B) forms of the S. alaskensis ectoine hydroxylase. The ribbon representations of the catalytic core of the ectoine hydroxylase shown in C and D are both based on a crystal structure that contained the iron catalyst, the 2-oxoglutarate co-substrate, and the product of the EctD-catalyzed enzyme reaction, 5-hydroxyectoine (PDB code 4Q5O). In the structure shown in C, only the binding of the iron catalyst and the 2-oxoglutarate (2-OG) molecules are shown, and the structure shown in D highlights only the binding of the iron catalyst and the captured 5-hydroxyectoine (Ect-OH) molecule. Amino acids involved in ligand binding are represented as sticks.
Architecture of the 5-Hydroxyectoine-binding Site—The 5-hydroxyectoine molecule is bound within the active site of SaEctD, allowing the binding of the iron catalyst and of the 2-oxoglutarate co-substrate and the EctD-catalyzed enzyme reaction product, 5-hydroxyectoine. Amino acids involved in ligand binding are represented as sticks. In (B) the electron density of an omit map (calculated by the Phenix software) of the 2-oxoglutarate and 5-hydroxyectoine molecules captured by the active site of SaEctD is shown as stereo figure. The 2Fo-Fc map was contoured at 1.0 sigma. Both figures are based on data deposited in the PDB under accession code 4Q5O.

FIGURE 8. Architecture of the active site of the S. alaskensis ectoine hydroxylase. The crystal structure shown in A highlights the network of interactions allowing the binding of the iron catalyst and of the 2-oxoglutarate co-substrate and the EctD-catalyzed enzyme reaction product, 5-hydroxyectoine. The crystal structure shown in B highlights the network of interactions allowing the binding of the iron catalyst and of the 2-oxoglutarate co-substrate and the EctD-catalyzed enzyme reaction product, 5-hydroxyectoine. Amino acids involved in ligand binding are represented as sticks. In (B) the electron density of an omit map (calculated by the Phenix software) of the 2-oxoglutarate and 5-hydroxyectoine molecules captured by the active site of SaEctD is shown as stereo figure. The 2Fo-Fc map was contoured at 1.0 sigma. Both figures are based on data deposited in the PDB under accession code 4Q5O.

and B) and observed no significant differences between these two structures, thus reinforcing our conclusion (22) that the capturing of the iron ligand does not induce significant structural changes in the ectoine hydroxylase.

Architecture of the 2-Oxoglutarate-binding Site—In the crystals that we obtained by adding Fe(II)Cl₂, the co-substrate 2-oxoglutarate, and the reaction product 5-hydroxyectoine, the densities for the 2-oxoglutarate and 5-hydroxyectoine ligands were clearly visible. We first describe the coordination of the 2-oxoglutarate co-substrate and subsequently that of 5-hydroxyectoine by the EctD protein.

The 2-oxoacid group of 2-oxoglutarate is placed in close vicinity to the iron catalyst in the active site (Fig. 7C). Interactions with the side chains of Arg-256, Ser-247, and Phe-141 are key for 2-oxoglutarate binding (Fig. 7C). Furthermore, stabilizing interactions of this ligand are formed with the side chain of Arg-129. The precise configuration of the Arg-129 side chain is in turn stabilized through interactions with the side chains of Phe-93 and Asn-131 (Fig. 7C). Arg-129 and Asn-131 are part of the DSBH core; however, Phe-93 is positioned in a β-sheet flanking this fold. The Arg-129/Phe-93 and Arg-129/Asn-131 interactions thereby stabilize the conformation of the SaEctD protein as a whole. Loss of these interactions would probably lay the active site of SaEctD more open, possibly rendering it unable to bind any of its substrates in a coordinated fashion. The 2-oxoglutarate ligand within the active site is further stabilized by Phe-260, although this interaction appears to be not very strong because the distance between 2-oxoglutarate and the Phe side chain is almost 4 Å (Fig. 7C).

The architecture of the 2-oxoglutarate-binding site of the SaEctD protein is similar to that found in crystal structures of many non-heme containing iron(II) and 2-oxoglutarate-dependent dioxygenases (38-40). It also corresponds closely to that suggested by structural comparison and modeling studies of the ligand-free VStEctD protein (43, 44).

Architecture of the 5-Hydroxyectoine-binding Site—The 5-hydroxyectoine molecule is bound within the active site of SaEctD slightly above the three residues (His-144, His-245, and Asp-146) forming the 2-His-1-carboxylate facial triad and in such a way that the hydroxy group at C5 in the pyrimidine ring points toward the iron catalyst (Fig. 7D). It is also positioned in close vicinity to the 2-oxoglutarate co-substrate (Fig. 8A). Hence, the spatial orientation of the 5-hydroxyectoine molecule is such as one would expect after the EctD enzyme has completed the hydroxylation of its substrate ectoine (27, 33, 43). The electron densities of the iron catalyst, the 2-oxoglutarate co-substrate, and the reaction product 5-hydroxyectoine are reasonably well resolved in the 2.56 Å SaEctD crystal structure (Fig. 8B).

Interactions of the EctD protein with the ring structure of 5-hydroxyectoine and its methyl and carboxyl groups position and stabilize this molecule within the active site (Fig. 7D). The side chains of Gln-127, Trp-150, and Arg-280 are directly involved in these interactions; a hydrogen bond to the backbone of Leu-284 and a weak interaction with the side chain of Thr-149 (distance of 3.9 – 4.0 Å) provide additional stability for the binding of the ligand (Fig. 7D). Arg-129 interacts with the side chain of Gln-127 and therefore provides further stability (Fig. 5A). In addition, Trp-150, a residue that belongs to the ectoine hydroxylase signature sequence (22, 27, 43), makes contact with the pyrimidine ring of 5-hydroxyectoine (Fig. 7D). This residue seems to play a special role in ectoine/5-hydroxyectoine binding because its conservative replacement with either Phe or Tyr residues in the VStEctD enzyme yielded catalytically inactive protein variants (43). This finding suggests that the interactions with the second aromatic ring structure of Trp are important for ligand stabilization. The carboxyl group of 5-hydroxyectoine is coordinated through interactions with Thr-149, a residue of the signature sequence motif, and His-144, which is part of the iron-coordinating 2-His-1-Asp facial triad motif (Fig. 7D).

Molecular dynamics simulations were recently used to glean information about the possible location of the ectoine substrate within the crystal structure of the VStEctD protein (43). Despite the moderate affinity of this enzyme for its substrate ectoine (Kₘ = 5.9 ± 0.3 mM) (22), this modeling approach positioned the ectoine molecule to the same ligand-binding pocket that was revealed through the crystallographic analysis of the
Crystal Structure of the Ectoine Hydroxylase

SaEctD protein presented in this study (Fig. 7D). However, relative to the spatial orientation of the 5-hydroxyectoine ligand found in the experimentally determined SaEctD crystal structure (Figs. 7D and 8A), the ectoine molecule is flipped by 180° around its vertical axis when compared with its position in the in silico generated model, and it is also slightly tilted (43). In the SaEctD crystal structure, the carboxylate of the 5-hydroxyectoine molecule is stabilized via hydrogen bonds (Figs. 7D and 8A), although such stabilizing interactions are missing in the VsEctD in silico model (43). Interactions with the carboxylate of either ectoine or 5-hydroxyectoine are important for high affinity ligand binding by solute receptor proteins operating in conjunction with either ABC or TRAP transport systems for these compounds (65–67). We therefore surmise that the SaEctD crystal structure in complex with 5-hydroxyectoine (Fig. 8A) also reflects the configuration and spatial position of the ectoine substrate when it is captured in the active site by the ectoine hydroxylase prior to catalysis.

Structure-guided Mutagenesis of 5-Hydroxyectoine-contacting Residues—The binding of the iron catalyst and the 2-oxoglutarate co-substrate in non-heme-containing dioxygenases is mediated by structurally well conserved motifs (37–40), and these are also present in the VsEctD (22, 44) and SaEctD proteins (Fig. 7, B and C). The corresponding residues in the VsEctD protein (Fig. 3) have already been targeted by site-directed mutagenesis and inevitably yielded mutant EctD proteins that were either strongly impaired in their enzyme activity or were catalytically inactive (43). In view of the fact that the corresponding iron- and 2-oxoglutarate-coordinating residues (Fig. 4, B and C) within the EctD enzyme family are practically completely conserved within a group of 433 inspected EctD-type proteins (22, 43), we have not repeated such experiments with the SaEctD protein. Instead, we have focused on those residues that contact the 5-hydroxyectoine molecule within the ligand-binding site of SaEctD (Fig. 7D).

Residues Gln-127, Thr-149, Trp-150, and Arg-280 (Fig. 7D) were individually substituted by an Ala residue; Leu-284 was not changed because it makes a backbone contact to 5-hydroxyectoine. Likewise, the 5-hydroxyectoine-contacting residues His-144 and Asp-146 were also not targeted by site-directed mutagenesis because their side chains are not only involved in 5-hydroxyectoine binding but are critical for binding of the iron catalyst as well (Fig. 7, B and D). The individual substitution of Gln-127, Thr-149, Trp-150, and Arg-280 by Ala residues yielded EctD variants that were either strongly impaired in their enzyme function (Thr-149/Ala and Arg-280/Ala), or almost completely catalytically inactive (Gln-127/Ala and Trp-150/Ala) (Table 3). Fully consistent with these results are data from a mutagenesis study of the VsEctD protein in which the two 5-hydroxyectoine-contacting residues corresponding to Gln-127 and Trp-150 in the SaEctD protein (Fig. 3) have been studied; their replacement yielded enzymatically inactive variants of EctD as well (43). Given the combined data from the site-directed mutagenesis studies of the SaEctD (Table 3) and the VsEctD (43) proteins, it is not unexpected that each residue involved in the binding of 5-hydroxyectoine is completely conserved in an alignment of 433 microbial EctD-type proteins (22, 43).

DISCUSSION

Members of the superfamily of non-heme-containing iron(II) and 2-oxoglutarate-dependent dioxygenases share similar overall structures and enzyme mechanisms, but they can catalyze a diverse set of oxidation reactions (37–41). The ectoine hydroxylase (EctD) belongs to this superfamily and mediates the stereo-specific hydroxylation of the compatible solute ectoine to form 5-hydroxyectoine (27, 68), which thereby gains novel stress-protective and function-preserving properties (11–15, 35, 36). The crystal structures of EctD from the extremophilic bacteria V. salexigens (44) and S. alaskensis (this study) are most closely related to the human hydroxylases PhyH and PHYD1A (55, 56) and to the microbial halogenases SyrB2, CytC3, and CurA (57–59). It is noteworthy that the substrates of the mentioned halogenases are all tethered via a thioester to the phosphopantetheine arms of acyl carrier proteins of polyketide synthases and nonribosomal peptide synthetases that function within assembly lines directing the synthesis of antibiotics and phytotoxins (57–59). In contrast, ectoine hydroxylases use a freely diffusible small molecule as their substrate (22, 26, 27).

Various quaternary conformations are found for enzymes belonging to the non-heme-containing iron(II) and 2-oxoglutarate-dependent dioxygenase superfamily (37–39, 62). Conventional size-exclusion chromatography (22) and HPLC-MALS analysis (Fig. 2) strongly suggest that ectoine hydroxylases obtained from various producer organisms are all dimers in solution. In the homodimer of the SaEctD crystal structure, the two monomers are oriented in a head to tail fashion and interact through loop regions (Fig. 4).

The crystallographic analysis of the SaEctD protein in complex with all its ligands (Fig. 8A) revealed that three residues contribute to iron binding, seven are involved in the binding of 2-oxoglutarate, and seven mediate the binding of 5-hydroxyectoine. Of these 15 residues, His-144 and Asp-146 are simultaneously involved in the binding of the iron catalyst and of 5-hydroxyectoine (Fig. 8A).

When one views the overall shape and surface structure of the crystallized EctD proteins as a whole, a deep cavity is immediately apparent in each of the monomers (Fig. 4B). The iron ligand, the co-substrate 2-oxoglutarate, and the EctD reaction product 5-hydroxyectoine (and by inference also the substrate ectoine) are all located in this cavity (Fig. 4B). As expected, all residues that we deem to be of functional importance for ligand binding and catalysis protrude into this cavity of the EctD protein (Figs. 4B and 8A).

Multiple cation/π interactions between the delocalized positive charges of the ectoine and 5-hydroxyectoine molecules (Fig. 1) and the side chains of aromatic residues of extracellular ectoine/5-hydroxyectoine-specific solute receptor proteins (EhuB, UehA, and TeaA) that operate in conjunction with either ABC or TRAP transport systems (65–67) are key determinants for high affinity binding of these tetrahedryoprimidines. These ligand-binding proteins possess $K_d$ values in the low micromolar range because they need to scavenge ectoines from scarce environmental resources for use either as osmoprotectants or as nutrients (65–67). Ectoine hydroxy-
Crystal Structure of the Ectoine Hydroxylase

JOURNAL OF BIOLOGICAL CHEMISTRY 29581

Furthermore, our crystallographic study might also inspire experiments to improve the moderate catalytic efficiencies of ectoine hydroxylases (22) through targeted mutagenesis or in vivo evolution experiments to enhance their industrial use.

Acknowledgments—We thank Jochen Sohn for excellent technical assistance in the overproduction and purification of the EctD protein and Georg Lentzen and Irina Bagyan (bitop AG) for their kind gifts of ectoine and 5-hydroxyectoine. We thank the staff of the P13 and P14 beamlines at EMBL, Hamburg, Germany, for their kind support during crystal screening. We acknowledge the European Synchrotron Radiation Facility for provision of synchrotron radiation facilities (ID23eh2). We greatly appreciate the expert help of Vickie Koogle in the language editing of our manuscript and thank Lutz Schmitt for helpful discussions and the kind support for this project. E. B. greatly valued the hospitality of Tom Silhavy during a sabbatical at the Department of Molecular Biology, Princeton University (Princeton, NJ). N. W. and E. B. are very grateful to Rolf Thauer for his continued support.

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