Structural and functional analysis of an L-serine O-phosphate decarboxylase involved in norcobamide biosynthesis

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Structural diversity of natural cobamides (Cbas, B12 vitamers) is limited to the nucleotide loop. The loop is connected to the cobalt-containing corrin ring via an (R)-1-aminopropan-2-ol O-2-phosphate (AP-P) linker moiety. AP-P is produced by the L-threonine O-3-phosphate (L-Thr-P) decarboxylase CobD. Here, the CobD homolog SMUL_1544 of the organohalide-respiring epsilon-proteobacterium Sulfurospirillum multivorans was characterized as a decarboxylase that produces ethanolamine O-phosphate (EA-P) from L-serine O-phosphate (L-Ser-P). EA-P is assumed to serve as precursor of the linker moiety of norcobamides that function as cofactors in the respiratory reductive dehalogenase. SMUL_1544 (SmCobD) is a pyridoxal-5'-phosphate (PLP)-containing enzyme. The structural analysis of the SmCobD apoprotein combined with the characterization of truncated mutant proteins uncovered a role of the SmCobD N-terminus in efficient L-Ser-P conversion.

Keywords: cobamides; ethanolamine phosphate; norcobamide biosynthesis; serine phosphate decarboxylase

Abbreviations
PLP, pyridoxal-5'-phosphate; TEV, tobacco etch virus.
Typhimurium strain LT2 (henceforth termed S. enterica). Three enzymes were found to be involved in this pathway (Fig. 2) [14–16]. Initially, l-threonine (l-Thr) is phosphorylated by the l-threonine kinase PduX (Enzyme Commission number EC 2.7.1.177) and l-threonine O-3-phosphate (l-Thr-P) is formed. Subsequently, l-Thr-P is decarboxylated by the l-threonine O-3-phosphate decarboxylase CobD (EC 4.1.1.81) yielding AP-P, which is attached to cobyric acid by the cobamidase phosphate synthase CbiB (EC 6.3.1.10). The difference in the NCba linker biosynthesis in S. multivorans that leads to the incorporation of EA-P

Fig. 1. Structure of vitamin B₁₂ in comparison to norpseudovitamin B₁₂ isolated from Sulfurospirillum multivorans. The prefix nor- refers to the absence of the methyl group at C176, while the prefix pseudo- refers to the presence of an adeninyl moiety (framed in the norpseudovitamin B₁₂ structure) rather than a 5,6-dimethylbenzimidazolyl moiety (framed in the vitamin B₁₂ structure) as lower ligand of the cobalt. The presence of the cyano-group (-CN) as upper ligand is a result of the cobamide purification, which is performed in the presence of cyanide. The cyanide replaces the natural upper ligand (e.g., a 5-deoxyadenosyl-, methyl-, or hydroxyl group).

Fig. 2. Biosynthesis and incorporation of the linker moiety in cobamides. Ado, S’-deoxyadenosyl moiety; l-Thr, l-threonine; l-Thr-P, l-threonine phosphate; AP-P, aminopropanol phosphate; l-Ser-P, l-serine-phosphate; EA-P, ethanolamine phosphate.

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remains unknown so far. The organism possesses a single large gene cluster for the anaerobic biosynthesis of norpseudo-B$_{12}$ (adeninyl-norcobamide, Ade-NCba) (Fig. 3) [17]. While a gene with homology to pduX encoding an $\alpha$-threonine kinase is not present in the S. multivorans genome, open reading frames encoding a CbiB homolog and a putative cobD gene (SMUL_1544) have been identified in the gene cluster for Ade-NCba biosynthesis. A previous study on SMUL_1544 revealed that the gene product complements the $\Delta$cobD growth defects in an S. enterica mutant strain [18]. Moreover, Ade-NCba rather than adeninyl-cobamide (Ade-Cba, pseudo-B$_{12}$) was produced by the S. enterica $\Delta$cobD mutant strain expressing the SMUL_1544 gene. This result already indicated a role of SMUL_1544 in EA-P production. Furthermore, isotopic labeling of the Ade-NCba linker moiety in S. multivorans cells cultivated in the presence of $\alpha$-[3-$^{13}$C] serine pointed toward $\alpha$-serine, most probably $\alpha$-serine O-phosphate (\textit{$\alpha$}-Ser-P) as precursor of EA-P [18].

The $\alpha$-Thr-P decarboxylase of S. enterica (ScCobD) contains pyridoxal-5'-phosphate (PLP), the biological active form of vitamin B$_6$ [14,19]. This type of cofactor was found in a huge variety of enzymes, which are functioning in essential metabolic pathways [20]. The PLP-dependent enzymes catalyze various reactions including decarboxylations, transaminations, racemizations as well as $\beta$- and $\gamma$-elimination reactions. The cofactor is bound via a Schiff base linkage to a lysine residue inside the protein (internal aldimine) [21,22]. One exception to this rule of cofactor binding was identified in the GDP-4-keto-6-deoxy-D-mannose-3-dehydratase [23]. Based on the structural properties of the PLP-enzymes, these proteins are classified in five superfamilies with several subfamilies. ScCobD as well as SMUL_1544 belong to the aspartate aminotransferase superfamily and the sequence motifs of their PLP-binding sites are typical for the aminotransferase class 1 subfamily (Prosite: PS00105). The similarity to aminotransferases and the potential role of SMUL_1544 in the formation of EA-P in S. multivorans raised the question, whether SMUL_1544 functions as an $\alpha$-Ser-P decarboxylase in Ade-NCba biosynthesis. In the study presented here, SMUL_1544 was heterologously produced, purified, and biochemically characterized. Mutagenesis of the enzyme’s N-terminus uncovered its role in defining the substrate specificity. The three-dimensional structure of SMUL_1544 was solved and its similarity to CobD-like decarboxylases and $\alpha$-histidinol phosphate aminotransferases (HisC) underpinned the assumption that the enzyme represents an evolutionary link between both classes of enzymes.

Materials and methods

Genetic constructs

For the production of a fusion protein of SMUL_1544 and a C-terminal Streptag-tag sequence, the SMUL_1544 gene was cloned into the vector pASK-IBA-63c-plus (IBA GmbH, Goettingen, Germany). The respective coding sequence was amplified via polymerase chain reaction by the use of genomic DNA of Sulfurospirillum multivorans as template and the primers F1 and R1 (Table 1). The PCR fragment was cut with BspHI and Xhol and ligated into the expression vector cut with NcoI and XhoI. The resulting plasmid pSMUL_1544 was transformed via heat-shock into Escherichia coli DH5a for proliferation or into E. coli BL21 (DE3) for protein production. The mutant genes encoding the truncated variants of SMUL_1544 (SmCobD) were generated and cloned in a similar way by the use of the primers F2 to F6 in combination with the reverse primer R2 (Table 1). The cobD gene of Salmonella enterica was amplified by the use of primers F7/R2 and genomic DNA as template for the PCR reaction and the gene encoding HisC from E. coli K-12 was amplified by the use of primers F8/R3 and genomic DNA as template. The PCR fragment containing cobD was cut with NcoI and XhoI and the PCR fragment harboring hisC was cut with BspHI and Xhol. Both were cloned into the vector pASK-IBA-63c-plus using the NcoI and XhoI restriction sites yielding plasmid pSeCobD and pEcHisC, respectively. The point mutations in SMUL_1544 and ScCobD were constructed via overlap extension PCR [24] using the plasmids pSMUL_1544 or

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**Fig. 3.** Gene cluster for norcobamide biosynthesis in Sulfurospirillum multivorans.
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Table 1. Primers used in this study

| Oligonucleotide | Sequence* (5‘-3’) |
|----------------|------------------|
| F1 (SMUL_1544Strep) | AGCGTCATGATGATAACATGAATGC |
| F2 (Δα 2-6) | AGTTCATGACACGTAAACTCAATTT |
| F3 (Δα 2-11) | GCTATCATGATTAGAAGCGTTTCA |
| F4 (Δα 2-16) | AGCTCTCATGATGCTATAAACAGAA |
| F5 (Δα 2-21) | TCAGCTCATGATGCGGGAGTCTAG |
| F6 (Δα 2-29) | ATGCTCATGACCTGGAAATAATTTG |
| F7 (SeCobD) | AGCTCCATGGCGTTATTCAACAGCG |
| F8 (EcHisC) | AGCTTCATGACGACGTGACTATTAC |
| F9 (H26A_Sm) | GAAGGCGGGAAGTGCAAGTCAAGTA |
| F10 (A107E_Sm) | GAATGTCGACCTGGAAATATTACG |
| F11 (S242T_Sm) | TTAAGTTGCTCGACTAAAGATTTTCG |
| F12 (T215S_Sm) | TTTCCGCTGCCAAATAATTATGCGTTTC |
| F13 (HisSMUL_1544) | TGACTCGGCTTGATGACATGAAAG |
| R1 (SMUL_1544Strep) | TATG |
| R2 (SeCobD) | ACTGCTCGAGATCAGACGGGCTATA |
| R3 (EcHisC) | TGCACCTCGAGAACCTTTGACAGT |
| R4 (H26A_Sm) | CCACTATCCGCTGACTCCGCA |
| R5 (A107E_Sm) | CTGAAATATTTTCAGCTCCACATCC |
| R6 (S242T_Sm) | CCAGAATCTTTTATGGAGATT |
| R7 (T215S_Sm) | GGAACTGCGACATAATAATTCGTCAGCG |
| R8 (HisSMUL_1544) | GACCACTCTGACTTACATATCTT |

* Mutated codons are underlined.

pSeCobD as templates. For the crystallization of SMUL_1544, a non-affinity tagged enzyme was produced as follows. The SMUL_1544 gene was cloned into vector pTEV5 [25]. The gene was amplified by the use of primers F13 and R8 in a PCR reaction with genomic DNA of S. multivorans used as template. Both, the vector and the PCR product were cut with NheI and XhoI and the resulting fragments were ligated. Cloning the SMUL_1544 gene into pTEV5 allowed for the production of a fusion protein of SMUL_1544 and an N-terminal Histag. The identity of all genes subcloned and modified in this study was verified by DNA sequencing.

Purification of the recombinant proteins

Escherichia coli BL21 (DE 3) strains containing the various expression vectors were cultivated in lysogeny broth medium supplemented with 100 µg·mL⁻¹ ampicillin (2% inoculum). The cultivation was performed at 37 °C and 100 r.p.m. shaking under aerobic conditions. After the bacteria reached an OD at 550 nm of 0.5, protein expression was induced by the addition of 200 ng·mL⁻¹ anhydrotetracycline (Sigma, Taufkirchen, Germany). After 3 hours of incubation at 37 °C, the cells were harvested by centrifugation (8000 g, 10 min, 10 °C) and the pellets were stored at −20 °C. For cell disruption, the cells were resuspended in 50 mM Tris-HCl pH 7.9 (1 mL·g⁻¹ cells) containing cComplete™, EDTA-free Protease Inhibitor Cocktail (Roche, Mannheim, Germany). The resuspension was incubated at 37 °C, 30 min, and 500 r.p.m. in the presence of 10 mg·mL⁻¹ lysozyme and DNase I. Subsequently the lysozyme-treated cells were shock frozen in liquid nitrogen and then thawed at 37 °C and centrifuged at 100 g for 15 min. The crude extract was obtained by centrifugation of the sample (1 h, 16 000 g, 4 °C). The recombinant proteins were purified from the crude extract by the use of Streptactin affinity chromatography (IBA) according to the manufacturer’s instructions. In the case of the SMUL_1544 derivative with the N-terminal Histag, the E. coli BL21 strain harboring the pTEV5 construct with the SMUL_1544 gene was cultivated in LB medium containing ampicillin at 37 °C to an OD at 650 nm of 0.5. After induction of the gene expression by the addition of 0.3 mM IPTG, the cells were cultivated further for 22 h. The cells of a 1 L culture were harvested and resuspended in 30 mL Ni-NTA (nickel-nitrilotriacetic acid) binding buffer (500 mM NaCl, 50 mM Tris/HCl pH 7.9, 5 mM imidazole). Lysozyme was added (1 mg·mL⁻¹) together with DNase I and phenylmethylsulfonyl fluoride (PMSF) and the cells were disrupted by sonication. The crude extract was centrifuged at 39 000 g for 20 min and the supernatant filtered (0.45 µm pore size). The sample was applied to column chromatography using the Ni-NTA His-Bind® Resin (Merck-Millipore, Darmstadt, Germany) in accordance to the manufacturer’s instructions. The pooled protein fractions containing the His-tagged SMUL_1544 were mixed with the tobacco etch virus (TEV) protease in a 1 : 20 ratio and incubated at room temperature for 3 h. The reaction mixture was dialyzed against the Ni-NTA binding buffer and applied to Ni-NTA column chromatography in order to remove uncleaved protein and the His-tagged TEV protease. This procedure was repeated twice and finally the protein was dialyzed against 50 mM Tris-HCl pH 7.9, 100 mM NaCl, 10% glycerol.

Enzymatic assays

The enzymatic decarboxylation assay was performed in accordance to the method described by Brusabber et al. [14]. In this assay, 2 mmol l-Ser-P or l-Thr-P and 0.05 mmol PLP were mixed in 0.5 mL reaction buffer...
(50 mM HEPES pH 7.9). The reaction was started by the addition of 50 μg of purified protein (e.g., SMUL_1544, ScCobD). The aminotransferase assay contained 2 mmol L-His-P, 2 mmol 2-oxoglutarate, and 0.05 mmol PLP in 0.5 mL reaction buffer (50 mM HEPES pH 7.9). Negative controls were included in all measurement series. If not stated otherwise, the reaction mixtures were incubated at 37 °C for 1 h and the reaction was stopped by exposing the reaction mixture to 100 °C for 5 min. After cooling down on ice, the samples were centrifuged (5 min at 16 000 g) and 450 μL of the supernatant underwent derivatization and was subsequently applied to the analysis via HPLC on a Knauer Smartline machine coupled to a diode array detector. For derivatization, the samples were mixed with 100 μL 0.5 mM potassium borate and 50 μL derivatization solution (70 μM o-phenaldialdehyde (oPA), 140 μM mercaptoethanol, 22 μL methanol, 5 mM potassium borate pH 9.2). After 2 min of incubation at room temperature, 100 μL of the derivatization mixture was transferred into 500 μL washing solution [50 mM sodium acetate pH 6.1, 20% (v/v) methanol]. Finally, 50 μL of this mixture was analyzed via HPLC. The stationary phase was a Chromolith® Performance RP-18e, 100-4.6 mm column (Merck) equipped with a Chromolith®, RP-18e, 5-4.6 mm guard column. The mobile phases were (A) tetrahydrofuran : methanol : 50 mM sodium acetate pH 5.9 (ratio 1 : 2 : 97) and (B) methanol : 50 mM sodium acetate pH 5.9 (ratio 80 : 20). The flow rate was 0.7 mL min⁻¹ and the column temperature was set to 30 °C. The absorption was recorded at 340 nm. The gradient was composed as follows: 100% A for 5 min, 100–75% A in 15 min, 75–0% A in 5 min, 0% A for 5 min, 0–100% A in 5 min, and 100% A for 10 min. L-Ser-P, L-Ser, l-Thr-P, l-Thr, EA-P, ethanolamine, aminopropanol, l-His-P, and l-glutamate were purchased from Sigma. The AP-P standard was obtained from ScCobD-mediated decarboxylation of l-Thr-P and subsequent purification of the reaction product via HPLC. The enzyme activities were calculated from the reduction in substrate concentration over time. For the dephosphorylation of the substrates or reaction products, the reaction mixtures of the enzymatic assays were treated with alkaline phosphatase as follows: 200 μL of the reaction mixtures was combined with 47 μL buffer [100 mM CHES pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 1% (v/v) Tween 20] and 3 μL alkaline phosphatase. After 1 h incubation at 37 °C, the reaction was stopped (5 min, 100 °C) and analyzed via HPLC as described above.

Determination of PLP-content
To determine the saturation of the purified enzymes with PLP, a protein solution (concentration: 11 μM protein referring to the monomeric forms) in 100 μL 50 mM HEPES pH 7.9 was prepared. The enzyme solutions were mixed with 100 μM PLP and incubated for 5 min at room temperature. Subsequently, the samples were concentrated in Vivaspin® 10 Ultrafiltration Units (Sartorius, Göttingen, Germany). The concentration of unbound PLP was reduced to a minimum (< 0.4 μM) by repeated concentration and dilution of the sample in 50 mM HEPES pH 7.9. The resulting sample was applied to UV/Vis-spectroscopy in a Varian Cary 100 UV/Vis Spectrophotometer (Agilent, Waldbronn, Germany) and the absorbance at 388 nm (PLP) and 280 nm (protein) was monitored in a quartz glass cuvette (d = 1 cm). The concentration of PLP was calculated using the molar extinction coefficient of PLP at 388 nm (ε = 4.9 mM⁻¹ cm⁻¹). The quotient Abs (280 nm)/Abs (388 nm) was calculated and compared to the result obtained for the purified SMUL_1544.

Crystallization of SMUL_1544
The purified protein was screened for initial crystallization conditions with a 144-condition sparse-matrix screen developed in the Rayment laboratory. Single crystals were grown at 4 °C by hanging drop vapor diffusion. A volume of 2 μL of 8 mg·mL⁻¹ SMUL_1544 in 10 mM HEPES (pH 7.6), 50 mM NaCl was mixed with 2 μL of a well solution containing 100 mM bis-(2-hydroxyethyl) amino-tris(hydroxymethyl) methane (BIS-TRIS) (pH 6.0), 16% poly(ethylene glycol) methyl ether 2,000 (MEPEG 2K), and 200 mM potassium glutamate. Hanging droplets were nucleated after 24 h from an earlier spontaneous crystallization event using a cat’s whisker. Crystals grew to approximate dimensions of 200 μm × 200 μm × 300 μm within 15 days. The crystals were transferred to a cryoprotecting solution that contained 100 mM BIS-TRIS (pH 6.0), 16% MEPEG 2K, 220 mM potassium glutamate, and 20% glycerol and vitrified by being rapidly plunged into liquid nitrogen. SMUL_1544 crystallized in space group C121 with one chain in the asymmetric unit and the following unit cell dimensions: a = 141.5 Å, b = 90.6 Å, and c = 70.8 Å. Structural data for SMUL_1544 (SmCobD) have been deposited in the protein data bank (PDB) under accession number 6OUX.

Data collection and refinement
X-ray data for the SMUL_1544 were collected at 100 K on Structural Biology Center beamline 19BM at the Advanced Photon Source in Argonne, IL. Diffraction data were integrated and scaled with HKL3000 [26]. Data collection statistics are listed in Table 2. The structure of SMUL_1544 was determined using 1LC8 as molecular replacement search models in the program Phaser [27]. Final models were generated by alternating cycles of manual building and least-squares refinement using COOT, PHE- NIX, and REFMAC [28–30].
Results

Enzymatic decarboxylation of l-serine O-phosphate

In analogy to the use of l-threonine O-3-phosphate (l-Thr-P) as precursor for the (R)-l-aminopropan-2-ol O-2-phosphate (AP-P) linker moiety of cobamides (Cbas), it was proposed earlier that l-serine O-phosphate (l-Ser-P) might serve as the precursor of the ethanolamine O-phosphate (EA-P) linker moiety in norcobamides (NCbas) [9]. EA-P might be produced from l-Ser-P via decarboxylation catalyzed by SMUL_1544 in Sulfurospirillum multivorans [18]. In order to proof this assumption, the SMUL_1544 gene was heterologously expressed, purified via affinity chromatography (Fig. S1), and applied in a decarboxylation assay [14]. After incubation with l-Ser-P or l-Thr-P as substrate and subsequent derivatization, the reaction mixtures were analyzed by HPLC coupled to photometric detection (Fig. S2). SMUL_1544 decarboxylated l-Ser-P to EA-P with a specific activity of 3.4 ± 0.7 nkat-mg⁻¹ protein. The HPLC elution profile of the substrate l-Ser-P showed two signals, which were both assigned to the phosphorylated amino acid by treatment with alkaline phosphatase. The dephosphorylation of l-Ser-P resulted in a single signal with a retention time identical to that of the l-serine standard (Fig. S3). The purified SMUL_1544 enzyme was also tested for its ability to convert l-Thr-P. Indeed l-Thr-P was decarboxylated and AP-P was formed with a conversion rate 80% lower (0.7 ± 0.4 nkat-mg⁻¹ protein) compared to the velocity monitored with l-Ser-P. SMUL_1544 did not decarboxylate l-serine or l-threonine.

The conversion of l-Ser-P and l-Thr-P by SMUL_1544 raised the question whether the standard-type l-Thr-P decarboxylase of Salmonella enterica (ScobD) also decarboxylates l-Ser-P. To provide an answer, ScobD was heterologously produced, purified, and tested with l-Ser-P as substrate. EA-P was generated by ScobD from l-Ser-P with a conversion rate that was 32% lower (1.6 ± 0.2 nkat-mg⁻¹ protein) compared to the activity measured with its preferred substrate l-Thr-P (5.0 ± 0.5 nkat-mg⁻¹ protein; Fig. S4).

### Table 2. Data collection and refinement statistics

|                      | Os79-UDP-open |
|----------------------|--------------|
| Space group          | C121         |
| Unit cell dimensions (Å) | a = 141.4; b = 90.6; c = 70.9 |
| Data collection site | Argonne 19BM |
| Wavelength (Å)       | 0.979        |
| Resolution range     | 50–1.94 (1.97–1.94) |
| Reflections: measured | 249 056     |
| Reflections: unique  | 55 968       |
| Redundancy           | 4.0 (3.2)    |
| Completeness (%)     | 99.5 (99.4)  |
| Average I/|/A            | 16.1 (2.0)   |
| Rmerge (%)           | 7.4 (5.0)    |
| Rwork                | 18.9         |
| Rfree                | 24.9         |
| Rmergeoverall        | 19.2         |
| Protein atoms        | 6028         |
| Water molecules      | 499          |
| Average B factors (Å²) | 25.3        |
| Ramachandran (%)     | 97.0         |
| Most favored         | 2.2          |
| Allowed              | 0.8          |
| Disallowed           | 0.02         |
| RMS deviations       | 2.0          |

a Values in parenthesis are for the highest resolution shell.

b \( R_{merge} = \frac{\sum(|I_{obs}| - \langle I \rangle) \times 100}{\sum|I_{obs}|} \), where the average intensity \( \langle I \rangle \) is taken over all symmetry equivalent measurements and \( I_{obs} \) is the measured intensity for a given observation.

c \( R_{factor} = \frac{\sum|F_{obs}| - |F_{calc}| \times 100}{\sum|F_{obs}|} \), where \( R_{work} \) refers to the \( R_{factor} \) for the data utilized in the refinement and \( R_{free} \) refers to the \( R_{factor} \) for 5% of the data that were excluded from the refinement.

The SMUL_1544 crystal structure

For the determination crystallization of the enzyme, the protein was purified via affinity chromatography and the affinity-tag was cleaved off proteolytically. A dimeric arrangement of SMUL_1544 was observed in the crystals, which was in coincidence with the dimerization of ScobD [19]. When SMUL_1544 and ScobD monomers were aligned (Fig. 4), the two protein structures overlapped with a root-mean-squared deviation (RMSD, calculated over 217 C-alpha atoms) of 1.3 Å.

SMUL_1544 is larger compared to ScobD (392 vs. 364 amino acids) because of an extended N-terminus as it was unraveled by the structural alignment (Fig. 4). Based on a sequence comparison (Fig. S5), it was deduced that the lysine K243 of SMUL_1544 might form the internal aldimine with pyridoxal-5’-phosphate (PLP), the essential cofactor of the decarboxylase. The residue was aligned to K216 (Fig. S5, red), which fulfills this function in the l-Thr-P decarboxylase ScobD [19,31]. The active site in both enzymes is located in a central cavity of the protein. The cofactor PLP and the substrate are held in place by a hydrogen bonding network established between the protein environment and mainly the phosphoryl groups of both compounds. The residues Y56, E85, T86, S213, T215, and R224 of ScobD interact with
the phosphoryl group in PLP via hydrogen bonding (Fig. S5, green). Two residues involved in PLP binding are exchanged in SMUL_1544. Alanine 107 of SMUL_1544 replaces E85 of SeCobD and S242 replaces T215. While the residue T215, located close to the Schiff base-forming K216 of SeCobD, was found to be highly conserved among CobD homologs, E85 is frequently replaced by threonine, serine, alanine, or asparagine (Interpro: IPR005860). Hence, the presence of S242 might reflect the altered substrate specificity of SMUL_1544. Two amino acids of SeCobD form hydrogen bonds with the pyridine ring of PLP (N157 and D185) (Fig. S5, grey) and N157 additionally binds the phosphoryl group of L-Thr-P. These amino acids are highly conserved among both, the CobD homologs and L-histidinol phosphate aminotransferases (HisC). Identical amino acid residues are also present in the SMUL_1544 amino acid sequence, namely N180 and D207. In contrast, SeCobD N32 (Fig. S5, grey) and N157 additionally binds the phosphoryl group of L-Thr-P. These amino acids are highly conserved among both, the CobD homologs and L-histidinol phosphate aminotransferases (HisC).

Another two other amino acid residues binding the phosphoryl group of the substrate in SeCobD, the C-terminal arginines (R323 and R337 in SeCobD) (Fig. S5, yellow), are also present in SMUL_1544 (R354 and R368; Fig. 5B).

The residues binding the phosphate group of l-Thr-P in SeCobD are conserved in the structure of SMUL_1544, except for the N-terminal histidine and glycine (H8 and G9 in SeCobD; Fig. 5B and Fig. 4, yellow). The histidine (H8, Fig. 5B) is replaced by a glycine (G26, not depicted in Fig. 5B) in the SMUL_1544 structure, which does not interact with the substrate. Instead of a glycine (G9, not depicted in Fig. 5B) that is binding the phosphate group of L-Thr-P in SeCobD, a serine (S25, Fig. 5B) is located at the same position in the structure of SMUL_1544. The hydrogen bond established between the protein backbone at the respective position and the substrate might not be affected by this exchange (Fig. 5C). The serine residue at position 25 is neighboring a histidine at position 26 in SMUL_1544 (Fig. 5C and Fig. 4, yellow). The amino acid residue H26 is 8 Å away from the phosphate of the substrate, which is too far for any interaction. However, when the histidine was exchanged by an alanine in SMUL_1544, the resulting mutant protein showed a decreased enzyme activity (Fig. 6). Changing H26 to an alanine could have an effect on the bond angles in the backbone and might...
disrupt the hydrogen bond between the backbone amide of S25 and the phosphate. Hence, H26 indeed plays a pivotal role for the decarboxylation activity of the enzyme. The binding of PLP to the SMUL_1544 (H26A) mutant protein was not affected.

Two amino acids responsible for the coordination of the phosphate group of PLP in \(\text{SeCobD} \) are altered in SMUL_1544 (Fig. S5, green). The glutamate E85 of \(\text{SeCobD} \) is replaced by A107 in SMUL_1544 and T215 by S242. The hydrogen bond between E85 and the phosphate of PLP is with the backbone amide and thus the alanine substitution may not remove this hydrogen bond (Fig. 5D). As T215 and S242 possess a hydroxyl group, this substitution does not necessarily remove the hydrogen bond, too. However, the structure reveals that the hydroxyl of the serine has been rotated to where the methyl group of the threonine was, not the hydroxyl group. It looks like the serine hydroxyl might be forming a hydrogen bond with the backbone amide of L252. One cannot be sure what the orientation of the serine hydroxyl would be if PLP was bound in the SMUL_1544 structure.

Both the amino acids, A107 and S242 in SMUL_1544, were genetically replaced by their respective counterpart. The exchange of A107 by glutamate resulted in a strong decrease in the amount of recombinant protein produced in the expression host and the purification of the mutant protein failed. The replacement of the S242 by threonine resulted in an enhanced conversion of \(\text{l-Thr-P} \) and a decreased enzyme activity with \(\text{l-Ser-P} \). The substrate preference of SMUL_1544 was not completely reversed, but the S242T variant displayed similar conversion rates for \(\text{l-Ser-P} \) (2.0 ± 0.4 nkat·mg\(^{-1}\) protein) and \(\text{l-Thr-P} \) (2.6 ± 0.6 nkat·mg\(^{-1}\) protein), which supports the assumption that S242 plays a role in defining the substrate range of the enzyme. This is a very interesting result, which cannot be explained readily from the structure. The replacement of T215 in \(\text{SeCobD} \) by a serine had no positive effect on the \(\text{l-Ser-P} \) conversion rate of this \(\text{l-Thr-P} \) decarboxylase. The T215S variant of \(\text{SeCobD} \) displayed very low activities for both, \(\text{l-Ser-P} \) and \(\text{l-Thr-P} \) (< 0.2 nkat·mg\(^{-1}\)). A spectroscopic analysis of purified \(\text{SeCobD} \) T215S uncovered that < 25% of the protein molecules contained a PLP cofactor. This finding indicated an improper binding of the cofactor to the mutant protein.

**Truncation of the N-terminus of SMUL_1544**

A substantial difference between SMUL_1544 and \(\text{SeCobD} \) is found in the N-terminal part of both proteins (Fig. 4). A series of five truncated variants was generated to test, whether the extended N-terminus of SMUL_1544 influences the substrate range of the enzyme (Fig. 6A). The largest deletion comprising 28
amino acids resulted in a mutant protein with a total length similar to ScCobD (364 amino acids). All mutant proteins were heterologously produced, purified, and analyzed in the decarboxylase assay. While the absence of the first five amino acids had no impact on the conversion rate of L-Ser-P, the enzyme activity was reduced by about 30% when 10, 15, or 20 amino acids were removed (Fig. 6B). The deletion of 28 amino acids prevented L-Ser-P almost completely. The L-Thr-P decarboxylation rate did not change substantially in all cases; however, the largest deletion (Δaa 2-29) also completely impaired the L-Thr-P conversion (Fig. 6B). These results uncovered a specific need for the last eight amino acids of the N-terminal stretch (N22AGSHPS29) for the functionality of the enzyme. This sequence includes S25 that has a function in binding of the substrate (Fig. 5C). The UV/Vis-spectroscopic analysis (ratio A280 nm/A388 nm) of the truncated SMUL_1544 protein lacking 29 amino acids in comparison to the wild-type revealed an unaffected saturation with the PLP cofactor.

**Phylogenetic affiliation of SMUL_1544**

Four amino acid residues in the active site cavity are indicative for L-Thr-P decarboxylases (H8, T86, F108, and W246 in ScCobD), but not present in the related L-histidinol phosphate aminotransferases [31]. These amino acids were proposed to prevent L-histidinol phosphate (L-His-P) from binding. The HisC-like aminotransferase of E. coli possesses the residues Y20, D85, Y110, and Y243 at the respective positions and binds L-His-P [32]. While the N-terminal histidine (H8 in ScCobD) is not present in SMUL_1544, the other three amino acids that were assigned to CobD homologs are conserved (T108, F130, and W272 in SMUL_1544). This fact is in good agreement with the results of the enzyme activity measurements described above that clearly showed the decarboxylase function of the enzyme.

In order to visualize the phylogenetic affiliation of SMUL_1544 in the aspartate aminotransferase family of PLP-dependent enzymes, a phylogenetic analysis with various CobD and HisC homologs was conducted (Fig. 7). Besides CobD homologs and HisC homologs, bacterial aspartate aminotransferases (AAT, EC 2.6.1.1) were included [33,34]. The latter group formed a single branch. SMUL_1544 clustered with the putative aminotransferases of Campylobacter jejuni (WP_052797156.1), Pedobacter steynii (WP_074605868.1), or Arcobacter butzleri L354 (KLE11288.1). SMUL_1544 displayed an elevated amino acid sequence identity of 46.2%, 46.6%, and 47.4%, respectively, with these proteins. The phylogenetic relationship of this group to the biochemically characterized HisC protein of E. coli K-12 (GenBank: CQR81539.1; [32,35] as well as the putative HisC proteins of S. enterica (GenBank: GAR68067.1), or S. multivorans (GenBank: AHJ11577.1) is closer compared to the group of CobD-like decarboxylases. The group of CobD homologs showed a sequence identity of 30–80%, but <30% to the HisC-like proteins. Aside from this, all CobD and HisC homologs shared <25% sequence identity with SMUL_1544. The aspartate aminotransferases included in the analysis have <21% sequence identity with the HisC and CobD homologs and thus occupied an even more distant position.

The high sequence similarity in SMUL_1544 with selected putative aminotransferases implied a functional overlap and might indicate that the protein represents a remnant of the transition between PLP-dependent aminotransferases and PLP-dependent decarboxylases. To test for the presence of aminotransferase activity, the purified enzyme was tested for the conversion of L-histidinol phosphate (L-His-P). When L-His-P and 2-oxoglutarate were applied as substrates for SMUL_1544, no formation of L-glutamate was observed, even after 24 h incubation time (Fig. S6). As a positive control, HisC of E. coli K-12 was
heterologously produced, enriched, and incubated with L-His-P and 2-oxoglutarate for 24 h at 37 °C. In this case, most of the substrate was consumed and product formation was detected (Fig. S6). This result proved the assay functional and supported the finding that SMUL_1544 does not possess L-His-P aminotransferase activity.

Discussion

Among the natural cobamides (Cbas, B12 vitamers), structural differences were observed, for example, norcobamides (NCbas) harbor an ethanolamine O-phosphate (EA-P) linker rather than an amino-propanol O-phosphate (AP-P) moiety between the corrin ring and the nucleotide moiety. Since the discovery of NCbas [9], the question for the specialty in the NCba biosynthetic pathway was raised. In the study presented here, the SMUL_1544 gene product, henceforth named SmCobD, was identified as L-serine O-phosphate (L-Ser-P) decarboxylase that forms EA-P from L-Ser-P and thereby most probably provides the building block for the NCba linker moiety. The SmCobD-coding gene (locus tag: SMUL_1544) is part of a large gene cluster that encodes for the NCba biosynthesis in Sulfuropirillum multivorans (Fig. 3) [17]. The genetic information for the organohalide respiration machinery in the epsilonproteobacterium S. multivorans was acquired via horizontal gene transfer. Almost all of the genes involved in NCba biosynthesis show the highest homology to genes found in Cba-producing Firmicutes, except the gene encoding for SmCobD. SmCobD might have been evolved from aminotransferase genes in epsilonproteobacteria.

As shown here, SmCobD is not limited to L-Ser-P conversion and decarboxylated L-threonine O-3-phosphate (L-Thr-P) to AP-P. S. multivorans lacks the genetic equipment for the formation of L-Thr-P [17]. However, the addition of L-Thr-P to growing cultures of the organism resulted in the formation of Cbas rather than NCbas by S. multivorans [18]. This previous finding already indicated the duality in the substrate specificity of SmCobD, which has been proven here. L-Thr-P is not a component of the synthetic medium [36] standardly applied for the cultivation of S. multivorans, thus the organism exclusively forms NCbas under laboratory conditions. Up to date, it remains uncertain whether the production of NCbas in S. multivorans represents an adaptation to the cofactor binding site of the cobamide-containing tetrachloroethene reductive dehalogenase (PceA) of the organism [37]. Data have been reported that indicated a flexibility of the enzyme in the utilization of NCbas and Cbas [18].

A previous analysis by Tavares et al. [38] did not detect any conversion of L-Ser-P by SeCobD, the classical L-Thr-P decarboxylase. However, in the study

Fig. 7. Phylogenetic analysis of different CobD, HisC, aspartate aminotransferases (AAT), putative aminotransferases (put. AT), and SMUL_1544 (red). The protein sequences were obtained from the NCBI database. A MUSCLE (v3.8) alignment [42,43] of the protein sequences was generated. The phylogenetic tree was created with MEGA7 [44] and the application of the Maximum likelihood method (1000 bootstrap replicates). The format refinement of the phylogenetic tree was done with FIGTREE v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).
presented here, enzymatic decarboxylation of \( \text{L-Ser-P} \) by \( \text{SeCobD} \) was observed with considerable rates. It is imaginable that all \( \text{L-Thr-P} \) decarboxylases are able to decarboxylate \( \text{L-Ser-P} \) and under conditions that lead to a shortage in \( \text{L-Thr-P} \) in a Cba-producing microbe, the utilization of \( \text{L-Ser-P} \) could complement the lack to some extent and NCbas are formed.

The origin of \( \text{L-Ser-P} \) as precursor of EA-P in \( S. \text{multivorans} \) cells was not traced back in this study. It could be formed from \( \text{L-serine (L-Ser)} \) by enzymatic phosphorylation. Alternatively, \( \text{L-Ser-P} \) might be recruited from the \( \text{L-Ser biosynthetic pathway} \) that includes the formation of \( \text{L-Ser-P} \) as an intermediate \cite{39}. For \( \text{L-Ser biosynthesis}, \) \( \text{3-phospho-D-glycerate} \) is converted to \( \text{L-Ser-P} \) by the enzymes SerA (phosphoglycerate dehydrogenase, \( \text{EC 1.1.1.95} \)) and SerC (phosphoserine aminotransferase, \( \text{EC 2.6.1.52} \)). \( \text{L-Ser-P} \) is dephosphorylated by SerB (phosphoserine phosphatase, \( \text{EC 3.1.3.3} \)) and \( \text{L-Ser} \) is formed. In a proteome study of \( S. \text{multivorans} \), the presence of SerA, SerC, and SerB was verified in cells producing NCbas \cite{40} making the availability of \( \text{L-Ser-P} \) as precursor for EA-P formation and NCba production in such highly probable cells.

The structure of \( \text{SmCobD} \) was solved here and despite the fact that the sequence identity between \( \text{SeCobD} \) and \( \text{SmCobD} \) is low (22%), the overall fold of both proteins was found to be similar. A pyridoxal-5'-phosphate (PLP)-loaded or substrate-bound \( \text{SmCobD} \) structure could not be solved in this survey. Most of the essential amino acids required for pyridoxal-5'-phosphate (PLP) and substrate binding in \( \text{SeCobD} \) are present also in \( \text{SmCobD} \) (Fig. 8). Based on the structural overlay of \( \text{SmCobD} \) and \( \text{SeCobD} \), it is hypothesized that the binding of \( \text{L-Thr-P} \) to \( \text{SmCobD} \) is disfavored by the presence of S25. The amino acid side chain of S25 is located in close distance to the position occupied by the methyl group of \( \text{L-Thr-P} \) in the \( \text{SeCobD} \) structure (Fig. 5B), which could result in steric hindrance and reduced affinity. In \( \text{SeCobD}, \) a glycine is replacing the serine.

The most obvious difference between both structures is the extended N-terminus of \( \text{SmCobD}, \) whose role was investigated by generating truncated mutant proteins. Although the first 20 amino acids did not appear to be involved in the formation of the substrate binding pocket, the stepwise removal of the N-terminus had a negative impact on the \( \text{L-Ser-P} \) conversion rate. This effect might be caused by a conformational change induced in the remaining part of the N-terminus (amino acids 22-29) that directly interacts with the substrate. It has to be noticed that the enzymatic decarboxylation of \( \text{L-Thr-P} \) was not affected to the same extent by the truncations. The assumption that small changes in the active site structure of \( \text{SmCobD} \) can interfere with enzyme function was also corroborated by the effect of the H26A amino acid exchange. The exchange resulted in the complete loss of enzyme activity, most probably due to an effect on the neighboring S25 that presumably binds the substrate (Fig. 5C).

A replacement of T215, which is involved in PLP binding in \( \text{SeCobD}, \) by a serine resulted in an unstable protein that did not efficiently bind PLP. When the unique S242, that occupies the same position in \( \text{SmCobD}, \) was replaced by a threonine, an enzyme variant was generated with similar conversion rates for both substrates. Hence, the presence of S242 indirectly supports the efficient \( \text{L-Ser-P} \) conversion. The phylogeny of \( \text{SmCobD} \) showed the highest similarity to putative, but uncharacterized aminotransferases. These enzymes contain a serine residue at the position of the \( \text{SmCobD} \) S242 and most active site residues specific for CobD homologs are also present. Probably the putative aminotransferases with elevated sequence similarity possess \( \text{L-Ser-P} \) decarboxylase activity that might be required for other metabolic pathways such as the glycerophospholipid biosynthesis \cite{41} rather than NCba production.
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Author contributions

SK and TS conceived the study. SK performed the phylogenetic analyses, constructed the genetic variants, and biochemically characterized all proteins. KMW, AV, and MS crystallized the wild type protein and KMW performed the structural analyses. SK drafted the manuscript. SK, KMV, IR, and TS analyzed the data and finalized the manuscript. SK and TS conceived the study. SK performed the reductive dehalogenase of *Dehalospirillum multivorans* was norpseudo-B12, a new type of a natural corrinoid. *Helv Chim Acta* **86**, 3698–3716.

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Fig. S1. Purified SMUL_1544 (wild-type) and truncated variants (5 µg protein per lane) and purified SeCobD separated on a 12.5% SDS/PAGE, which were stained with Coomassie.

Fig. S2. Decarboxylation of L-Ser-P or L-Thr-P by SMUL_1544.

Fig. S3. HPLC elution profiles of L-serine (L-Ser) and ethanolamine (EA) standards (first trace), L-Ser-P treated with alkaline phosphatase (AP) (second trace), and L-Ser-P decarboxylated by SMUL_1544 and subsequently treated with AP (third trace).

Fig. S4. HPLC elution profiles of the reaction products with L-Ser-P or L-Thr-P as substrate in the absence or presence of SeCobD after treatment with AP.

Fig. S5. Pairwise sequence alignment of SeCobD (GenBank: AAC79515.1) and SMUL_1544 (NCBI Reference Sequence: WP_025344678.1).

Fig S6. HPLC elution profiles of aminotransferase reactions with L-His-P and 2-oxoglutarate.