METALLOPROTEINS

Structural basis for organohalide respiration

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Organohalide-respiring microorganisms can use a variety of persistent pollutants, including trichloroethene (TCE), as terminal electron acceptors. The final two-electron transfer step in organohalide respiration is catalyzed by reductive dehalogenases. Here we report the x-ray crystal structure of PceA, an archetypal dehalogenase from Sulfospirillum multivorans, as well as structures of PceA in complex with TCE and product analogs. The active site harbors a deeply buried non-pseudo-B12 cofactor within a nitroreductase fold, also found in a mammalian B12 chaperone. The structures of PceA reveal how a cobalamin supports a reductive haloelimination exploiting a conserved B12-binding scaffold capped by a highly variable substrate-capturing region.

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Several hundred RDase gene sequences de-posited in databases await testing for functionally and determination of the substrate spectrum. Low growth yields and the oxygen sensitivity of the RDases have hindered large-scale purification and biochemical characterization of RDases (1). Genetic manipulation of the bacterial isolates has thus far been difficult, and only recently was functional heterologous production of RDases reported, but it remains challenging (4). Structural data will help to resolve how RDases evolved, function, and specifically select the many different substrates, some of which have been present in the biosphere for less than a century.

Here we report the crystal structure of a reductive dehalogenase, PceA (5), of the microaerophilic epsilonproteobacterium Sulfospirillum multivorans (formerly Dehalospirillum multivorans) in an empty state; (ii) in the presence of TCE; (iii) in the presence of the cis-dichloroethene (cis-DCE) analog cis-dibromoethene (trans-DCE); and (iv) in the presence of iodide, a substitute for the leaving chlorine, at a maximum resolution of 1.6 Å (7). S. multivorans was isolated in the mid-1990s from activated sludge and possesses a flexible catabolism integrating numerous terminal reductases encoded in its genome (8). S. multivorans is able to couple the reductive dechlorination of PCE, TCE, or dibromoethene (DBE) to growth (9, 10) through its prototypical RDase PceA.

The 464 amino acids of PceA are structured in an N-terminal unit (residues 1 to 138), a non-pseudo-B12 binding core (residues 139 to 163) and possesses a flexible catabolism integrating numerous terminal reductases encoded in its genome (8). S. multivorans is able to couple the reductive dechlorination of PCE, TCE, or dibromoethene (DBE) to growth (9, 10) through its prototypical RDase PceA.

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Here we report the crystal structure of a reductive dehalogenase, PceA (5), of the microaerophilic epsilonproteobacterium Sulfospirillum multivorans (formerly Dehalospirillum multivorans) in an empty state; (ii) in the presence of TCE; (iii) in the presence of the cis-dichloroethene (cis-DCE) analog cis-dibromoethene (trans-DCE); and (iv) in the presence of iodide, a substitute for the leaving chlorine, at a maximum resolution of 1.6 Å (7). S. multivorans was isolated in the mid-1990s from activated sludge and possesses a flexible catabolism integrating numerous terminal reductases encoded in its genome (8). S. multivorans is able to couple the reductive dechlorination of PCE, TCE, or dibromoethene (DBE) to growth (9, 10) through its prototypical RDase PceA.

The 464 amino acids of PceA are structured in a compact α/β fold domain (Fig. 1A). The structure can be divided into an N-terminal unit (residues 1 to 138), a non-pseudo-B12 binding core (residues 139 to 163) and 216 to 323), an insertion unit (residues 164 to 215), an iron-sulfur cluster binding unit (residues 324 to 394) and a C-terminal unit (residues 395 to 464) (Fig. S1). Two protomers in the P41 asymmetric unit interact tightly to form a dimer with a twofold noncrystallographic symmetry. Two α helices of the non-pseudo-B12 binding core and one α helix of the N-terminal unit form a helical bundle with their symmetry mates. Along with extensive loop regions in the N- and C-terminal units, these bundles create the dimer interface. The interface covers 20% of the accessible surface area of the protomer, supporting a compact and stable dimeric arrangement. Using gel filtration, an apparent molecular mass of 89 kDa (Fig. S2) was determined for PceA purified from the membrane fraction, which agrees with a dimeric rather than a monomeric structure as reported previously for the soluble wild-type enzyme (5).

RDases lack obvious sequence similarities to other enzyme families, and the fold of PceA is unlike that of known corrinoid-dependent methyltransferases (11) or mutases (12). The most similar protein with clear homology found was methylmalonic aciduria cblC type with homocysteimurin (MMACHC) (13) (Fig. S3). MMACHC is a B12 trafficking chaperone essential for the formation of adenosyl- or methylcobalamin in humans by catalyzing the reductive removal of the upper axial ligands from cyanocobalamin and alkylcobalamins. Structural homology is limited to the B12/norpseudo-B12 binding core, which resembles the nitroreductase family fold (14) (Fig. S3). Consequently, RDases and MMACHC most likely evolved from a common ancestral B12 binding protein.

In addition to the non-pseudo-B12 cofactor, PceA also harbors two [4Fe-4S] clusters. Short distances between the two [4Fe-4S] clusters and the proximal [4Fe-4S] cluster and the Co bound to the corrin ring are expected to allow for a rapid electron transfer within a protein monomer (15) (Fig. 1B). The proximal [4Fe-4S] cluster is in van der Waals (vdW) contact distance to the C89 carbamoyl side chain and C8 of the corrin ring [for atom numbering, see (21)] with the carboxamide N84 being in hydrogen bond distance to a μ3-sulfido ligand of the [4Fe-4S] cluster. The two active sites of the PceA dimers are at a Co-Co distance of 42 Å without cofactors between them, indicating two independent catalytic units per dimer (Fig. 1A).
Both [4Fe-4S] clusters are within 6 Å of the enzyme surface, whereas norpseudo-B$_{12}$ is deeply buried in the structure. Most interactions between the cobalamin cofactor’s ring system and the protein matrix are due to hydrogen bonds between the cofactor and the norpseudo-B$_{12}$–binding core of PceA (Fig. 2). Binding of the phosphate and parts of the adenine group of norpseudo-B$_{12}$ is mediated by a loop in the iron-sulfur cluster binding unit. Co is not coordinated by adenine (base off), and the conformation of the linker is curled rather than extended as is typical for base-off B$_{12}$ (14, 16). Compared to the base-on conformation of isolated norpseudo-vitamin B$_{12}$ (2), several conformational changes, including a 90° rotation around the bond between phosphate and the C$^\prime$ of ribose and flip of the ribose, are needed to convert the base-on conformation into the base-off conformation found in PceA (Fig. 2). In comparison to the dimethylbenzimidazole moiety of B$_{12}$, the adenine moiety of norpseudo-B$_{12}$ has several hydrogen-bond acceptor and donor functions, which in addition to anchoring the cofactor allows solvent accessibility (Fig. 2, inset). C176 of the nucleotide loop packs closely against the adjacent $\beta$ sheet, and the methyl group found in B$_{12}$ at this position would force the loop to adopt a different conformation, which might explain the preference for norpseudo-B$_{12}$.

PceA contains CoII in the as-isolated state (2), for which an axial ligand is typically observed. Additional density is found above the Co, with a Co-X distance of 2.5 Å. The density has been tentatively assigned to a water molecule. Reduction to the catalytically relevant super-reduced CoI is probably facilitated by the weak axial ligation, making Co effectively tetracoordinated in the protein, and agrees with the elevated midpoint potential of the CoII/CoI transition of $\sim$380 mV (pH 7.5, versus a standard hydrogen electrode) (2).

Substrate access to the active site is restricted by a selection filter with the shape of a 3 × 5.5 Å “letterbox,” a gap made up of side chains from the conserved polar residues in proximity to the axial position of Co are depicted as white sticks, hydrophobic residues of the second ligand sphere as blue sticks. A 12 Å–long channel was identified as the substrate entry site to the active-site dome.

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**Fig. 1. Crystal structure of PceA.** (A and B) Overall structure of dimeric PceA. One protomer is shown in gray. The other protomer is colored as a conserved region, which binds the cofactors (green, residues 139 to 163 and 216 to 462), and a variable region, which contributes most of the substrate binding and selection function (blue, residues 1 to 138 and 164 to 215). Each PceA protomer harbors two cubane-type [4Fe-4S] clusters (proximal and distal, spheres) and norpseudo-B$_{12}$ (purple sticks). Supposed electron transfer pathways are indicated in (B), along with distances between the closest Fe atoms of the [4Fe-4S] clusters and Co of norpseudo-B$_{12}$. (C) Active-site cavity of PceA. Conserved polar residues in proximity to the axial position of Co are depicted as white sticks, hydrophobic residues of the second ligand sphere as blue sticks. A 12 Å–long channel was identified as the substrate entry site to the active-site dome.

**Fig. 2. Norpseudo-B$_{12}$ binding.** Potential hydrogen-bonding residues are colored according to their position within PceA: blue, N-terminal (residues 1 to 138); purple, norpseudo-B$_{12}$–binding core (residues 139 to 163 and 216 to 323); green, insertion unit (residues 164 to 215); yellow, [4Fe-4S]-cluster coordinating loop (residues 324 to 394). The 1$s$2mFo-DFc electron density map for norpseudo-B$_{12}$ and water molecules is shown as mesh. The inset shows the solvent-accessible adenine moiety. A comparison of corrinoid linker geometries is shown on the right: free norpseudovitamin B$_{12}$ (2), base on (Cambridge Crystallographic Data Centre: 217274, top), norpseudo-B$_{12}$ bound to PceA (middle), and base-off B$_{12}$ in MMACHC (14) (Protein Data Bank: 3SOM, bottom). The $\beta$ ligand is omitted for clarity.
the N-terminal and insertion units (fig. S4). After the filter, PCE and TCE have to pass through a 12 Å-long hydrophobic channel isolating the active site inside the core of the protein (Fig. 1C, arrow). The channel then expands to create the amphiphilic active site pocket at the β face of the corrin ring, where access to the cofactor itself is further restricted by a ring fence of side chains (fig. S4A). The cavity is predominantly lined by tryptophan and tyrosine residues, which define its shape and volume. Three adjacent polar residues, namely Tyr β 246, Arg β 305, and Asn γ 272 from the β2-helix binding core, are highly conserved (Figs. 2 and 3C). Of these Tyr β 246 is invariant, whereas some RDases have functionally conserved replacements with a lysine residue in place of Arg β 305 (fig. S5). Asn γ 272 is conserved in a subset of RDases. The residues are close to the corrin ring and point with their side chains into the active site pocket, positioning the phenolic hydroxyl group of Tyr β 246 in hydrogen-bonding distance to the guanidinium group of Arg β 305 (Fig. 3C).

To further analyze how substrate specificity and regioselectivity are determined by the protein structure, we analyzed the structure of PceA with its substrate TCE and the tribromoethene dehalogenation product cis-DBE. After soaking with TCE, additional density matching the shape of TCE was found in the active site pocket above the corrin ring (Fig. 3), concomitant with a reduction in the occupancy of the close-by water ligand. TCE binds with the dichlorinated carbon (Cl) facing the corrin ring. Judged by the shape of the electron density, TCE bound in two orientations with the lone chloride in cis and trans to the Co-facing chloride (Fig. 3A). The PCE/TCE binding pocket is restricted by vdW contacts to the surrounding aromatic side chains (fig. S4). Tyr β 246 is within hydrogen-bonding distance to the Cl substituent of TCE nearest to Co.

Reductive dehalogenation of TCE produces cis-DCE, whose binding site and orientation we revealed using cis-DBE, cis-DBE was used because the stronger anomalous scattering of Br as compared to Cl allowed identification of cis-DBE also at low occupancy. Two strong patches of density were observed in the same place as two Cl atoms in the minor orientation of TCE, marking the position of cis-DBE. The chloride atom of TCE nearest to Co and Tyr β 246 is unoccupied in the product complex (Fig. 3B and fig. S6).

The side chains in the substrate-binding pocket are tightly packed with little conformational freedom and probably disfavor the binding of molecules significantly larger than PCE or TCE by steric exclusion. Thus, the binding pocket provides a second gate for substrate selection. In contrast to the variable residues forming the channel entrance, aromatic residues within the pocket are partially conserved between different RDases but are dispersed in sequence (fig. S5). Two layers of control over substrate selection (the “letterbox” and the active site) could achieve the necessary selectivity within the wide range of organohalides converted by distinct RDases, which often exist within the same organism (5).

PceA is attached to the periplasmic side of the cytoplasmic membrane in S. multivorans cells (17). For both PceA monomers to function independently, the twofold noncrystallographic symmetry axis of the dimer should be perpendicular to the membrane plane in the PceA-PceB complex. This would agree with two principal orientations of our PceA structure on the membrane. Amino acid residues 411 to 431 (Fig. 4, red ribbon) show high flexibility in all crystals and could only be modeled in one out of six proteomes in an alternative (P21) crystal form, where α-helix 15 (fig. S1) is stabilized by a crystal contact. The disordered helices in both monomers are on the same face of the structure, and we speculate that this is the site of interaction with the PceB membrane anchor that is destabilized after complex dissociation in the purification process. Our proposed arrangement would locate the two electron entrance ports close to the membrane and the substrate channel pointing toward the periplasmic space (Fig. 4).

RDase catalysis involves a transfer of two electrons and a proton, while accommodating the dissociation of a chloride ion from the substrate. The initial step in dechlorination of PCE probably involves a dissociative electron transfer from CoI to PCE, resulting in the formation of a trichlorovinyl radical (18, 19) by chloride elimination, while the cofactor is returned to the CoIII state (18, 20, 21). The distance for this electron transfer between Co and Cl of TCE is 5.8 Å in our structure (Fig. 4).

The intermittent water (Fig. 3A) is depopulated in the TCE-bound active site only, suggesting that the β2-ligand water, 2.2 Å from the proximal chloride, is displaced by TCE. The same position was identified as a weak halide-binding site by a strong anomalous signal for iodide at 0.5 M concentration (fig. S6D) but the absence of such a signal at lower chloride concentrations. This would qualify the proximal chloride at CoI to the tightly enclosed distal chloride as the leaving group, ultimately yielding cis-DCE from the minor orientation of TCE displayed in Fig. 3A, consistent with the data obtained with the cis-DBE product analog.

Recombination of the trichlorovinyl radical with CoIII, forming an organometallic intermediate as proposed for free Br2 (22), is an attractive next step but would be disfavored in PceA because of...
the dense packing above the β face of the corrin ring (fig. S4A). The same steric constraints would disable an initial nucleophilic attack of Co577 on PCE. Instead, the short substrate-cofactor distances would allow the second electron transfer to occur toward C1 and could donate the required proton to neutralize the carbamion (26). Deprotonation of Tyr246 could be stabilized by the neighboring positive charge of Arg305. Equally, a role of Tyr246 in a radical (46) cannot be excluded.

REFERENCES AND NOTES

7. Materials and methods are available on Science Online.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1 to S6

Table S1

References (23–51)

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WORKING MEMORY

Medial prefrontal activity during delay period contributes to learning of a working memory task

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Cognitive processes require working memory (WM) that involves a brief period of memory retention known as the delay period. Elevated delay-period activity in the medial prefrontal cortex (mPFC) has been observed, but its functional role in WM tasks remains unclear. We optogenetically suppressed or enhanced activity of pyramidal neurons in mouse mPFC during the delay period. Behavioral performance was impaired during the learning phase but not after the mice were well trained. Delay-period mPFC activity appeared to be more important in memory retention than in inhibitory control, decision-making, or motor selection. Furthermore, endogenous delay-period mPFC activity showed more prominent modulation that correlated with memory retention and behavioral performance. Thus, properly regulated mPFC delay-period activity is critical for information retention during learning of a WM task.

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W orking memory (WM) is essential for cognition by allowing active retention of behaviorally relevant information over a short duration known as the delay period (1–3). Previous studies have shown that the prefrontal cortex (PFC) is crucial for WM, because perturbation of PFC activity impaired WM (3) and WM-related activity was observed during the delay period in neurons of dorsal-lateral PFC (DL-PFC) in primates and medial PFC (mPFC) in rodents (3–10). Nevertheless, the functional role of PFC delay-period activity in WM remains unclear. Memory retention and attentional control are leading candidates (3, 21, 22). However, PFC is also critical for other brain functions (11–14), including transcranial magnetic stimulation (15) and electrical stimulation (16). These roles cannot be distinguished by a delayed-response task, in which decision-making precedes the delay period (3, 12). In addition, traditional methods for perturbing neural activity (3), including transcranial magnetic stimulation (17) and electrical stimulation (16), do not provide the temporal resolution and cell-type specificity required for delineating the functional role of PFC delay-period activity in WM. We addressed these issues by using a WM task with a delay period designed to temporally separate memory retention from other functions (6, 9, 12, 20) and optogenetic approaches (27) to bidirectionally manipulate mPFC activity of excitatory and inhibitory neurons during the delay period.

Head-fixed mice were trained to perform an olfactory delayed nonmatch to sample (DNMS) task (Fig. 1A and B; fig. 5I; and movie S1), a modified version of the behavioral paradigms previously used in rats (19, 20). For each trial, an olfactory stimulus (ethyl acetate, EA, or 2-pentanone, 2P) was presented as the sample, followed by a delay period (4 to 5 s) and then a testing olfactory stimulus, either matched or nonmatched to the sample. Water-restricted mice were rewarded with water if they licked within a response time window in the nonmatch but not match trials (Fig. 1B and fig. S2). During the delay period, mice need to retain the information associated with the odor sample. The performance correct rate (referred to hereafter as performance), correct rejection rate, discriminability (d′), and lick efficiency steadily increased throughout the training, but there was a ceiling effect for the hit rate (Fig. 1C and D, and fig. S3). The potential involvement of visual, auditory, or somatosensory cues was excluded (fig. S4A). The potential involvement of visual, auditory, or somatosensory cues was excluded (fig. S4A). The potential involvement of visual, auditory, or somatosensory cues was excluded (fig. S4A). The potential involvement of visual, auditory, or somatosensory cues was excluded (fig. S4A). The potential involvement of visual, auditory, or somatosensory cues was excluded (fig. S4A).

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