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Heterologous expression, purification and cofactor reconstitution of the reductive dehalogenase PceA from *Dehalobacter restrictus*

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ABSTRACT

Organohalide respiration is used by a limited set of anaerobic bacteria to derive energy from the reduction of halogenated organic compounds. The enzymes that catalyze the reductive dehalogenation reaction, the reductive dehalogenases, represent a novel and distinct class of cobalamin and Fe-S cluster dependent enzymes. Until now, biochemical studies on reductive dehalogenases have been hampered by the lack of a reliable protein source.

Here we present an efficient and robust heterologous production system for the reductive dehalogenase PceA from *Dehalobacter restrictus*. Large quantities of Strep-tagged PceA fused to a cold-shock induced trigger factor could be obtained from *Escherichia coli*. The recombinant enzyme was conveniently purified in milligram quantities under anaerobic conditions by StrepTactin affinity chromatography, and the trigger factor could be removed through limited proteolysis. Characterization of the purified PceA by UV–Vis and electron paramagnetic resonance (EPR) spectroscopy reveal that the recombinant protein binds methylcobalamin in the base-on form after proteolytic cleavage of the trigger factor, and that 4Fe-4S clusters can be chemically reconstituted under anoxic conditions. This study demonstrates a novel PceA production platform that allows further study of this new enzyme class.

Introduction

In the 20th century, large quantities of halogenated organic compounds (organohalides) were released to the environment by industrial applications. Two such organohalides are tetrachloroethene (also known as perchloroethylene, PCE), widely used in the dry cleaning of fabrics and an excellent solvent for organic material and trichloroethene (TCE), a volatile anesthetic. The highly toxic and potential carcinogenic properties of these compounds and their stability were only later widely documented. In fact, their relative stability has made them amongst the most common groundwater and soil contaminants to be found [1,2].

Chemical clean up of these compounds has proved very difficult but interestingly a variety of anaerobic bacteria are able to detoxify organohalides during a process recently termed organohalide respiration [3]. In organohalide respiration, the organohalides serve as the terminal electron acceptor and are thus reductively dehalogenated. [3–6]. Bacterial species that are able to detoxify organohalides belong to both Gram-positive and Gram-negative groups and are usually strictly anaerobic [7]. The key enzyme that catalyzes the terminal electron transfer step leading to dechlorination is the reductive dehalogenase (RdhA) [8,9]. Recent genome sequences from organohalide respiring species have revealed some to contain up to 32 RdhA genes [10].

RdhAs constitute a new class of cobalamin and 4Fe-4S dependent enzymes [11,12]. Several RdhA enzymes have been characterized in terms of substrate specificity, cofactor content and steady-state kinetics. These studies represent a considerable undertaking since the host cells grow slowly, do not reach high cell density and require the presence of large amounts of organohalides for protein expression. With one exception [13], all RdhAs are monomeric (ranging from 35 to 65 kDa) and contain one cobalamin and two Fe-S clusters [9]. Arguably, the most extensive studied RdhAs are PceA from *Dehalobacter restrictus* [14,15], CprA from *Desulfitobacterium dehalogenans* [16] and PceA from *Sulfurospirillum multivorans* [17]. EPR studies have indicated that the cobalamin is bound in the base-off form, and established the presence of two 4Fe-4S clusters or one 4Fe-4S and one 3Fe-4S cluster. The enzymes have been reported to be oxygen-sensitive. In the case of PceA from *S. multivorans* the structure of the extracted cobalamin has been identified as norpseudocobalamin [18].

Due to the presence of a TAT signal sequence, it is assumed that RdhAs are transported into the periplasm [19]. Upon translocation, they are thought to associate with the membrane where they are anchored via a small transmembrane protein (RdhB) [20].

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The structure–function relationships for RdhA remain uncharacterized, as no structural model is available [21]. It is therefore of great interest to develop a recombinant RdhA expression and purification system, which can serve to facilitate further study. Hitherto, efforts aimed at achieving this have met with little success [22,23].

Here we present the first successful recombinant *Escherichia coli* production system, using the ~61 kDa reductive dehalogenase PceA from *D. restrictus*. Extensive screening of solubility tags and expression trials has revealed that a soluble form of PceA is obtained only when fused to the *E. coli* trigger factor (TF). Utilizing a C-terminal Strep-Tag II fused to TF-PceA, the protein could be recovered to >95% purity in a single affinity purification step. We were able to demonstrate that recombinant PceA can bind methylcobalamin and that the 4Fe-4S clusters can be fully chemically reconstituted under strict anaerobic conditions.

Material and methods

Construct design

The codon optimized *pceA* gene sequence, including its TAT recognition sequence from *D. restrictus* (GenBank accession number Q8GJ27), was synthesized by Eurofins MWG Operon. *PceA* was subsequently cloned into several different expression plasmids by the ligation independent In-Fusion™ technology [TaKaRa Bio Inc.]. Expression vectors were derived from the pOPIN series (Oxford protein production facility, OPPF) containing either a N-terminal His_6-tag (pOPINF), SUMO tag (pOPINS) or MBP tag (pOPINM) [OPPF Oxford, UK]. The *pceA* gene was inserted into these vectors using the restriction sites *HindIII* and *KpnI* (New England Biolabs). Similarly *pceA* was cloned into the pET50b(+) plasmid (Novagen) and the pCOLD TF plasmid (TaKaRa Bio Inc.). All constructs are under the control of an IPTG inducible *lacZ* operon. The pCOLD vector additionally contains a cold shock promoter (*cspA*), allowing induction of gene expression at low temperatures (15 °C) [24]. The Strep-Tag II was inserted downstream of *pceA* in the pCOLD TF vector using conventional cloning techniques.

Small-scale solubility screens

In all cases BL21 (DE3) cells (Merck) were transformed and used for expression trials in 100 mL cultures. Several different growth media (LB, TB, 2xYT and autoinduction media; all purchased from Formedium) were tested. Typically proteins were overexpressed (except for the autoinduction media) with 0.1 mM IPTG (Sigma) at temperatures of 25 °C, 18 °C or 15 °C (in case of the pCOLD TF) for 12–16 h. The 100 mL cultures were inoculated with 1% (v/v) of an overnight culture and grown at 37 °C, until mid-log phase, at which point the temperature was reduced and protein expression induced with IPTG. Soluble and insoluble fractions were prepared with BugBuster (Novagen) according to the manufacturer instructions and analyzed by SDS–PAGE.

Large-scale expression of the PceA from the pCOLD TF system

Cultures (usually 12 or 24 L of LB media) were inoculated with 1% (v/v) of an overnight culture. Cells were grown at 37 °C until mid log phase (0.6 $OD_{600\ nm}$). Overexpression of the trigger factor PceA fusion protein was induced at 15 °C, with the addition of 0.1 mM IPTG and 0.1 mM $FeCl_3$. After 12–16 h cells were harvested by centrifugation and stored at −20 °C until needed.

Anaerobic protein purification and cleavage of trigger factor with HRV 3C protease

Purification of TF-PceA was carried out under strict anaerobic conditions, with all buffers made anaerobic by sparging with nitrogen gas before use. Cell pellets were resuspended in buffer A (50 mM Hepes pH 8.0, 150 mM NaCl and 10% glycerol), supplemented with lysozyme, DNase, RNase (Sigma) and Complete EDTA free protease inhibitor cocktail tablets (Roche Diagnostics) and stirred for 30 min on ice under nitrogen atmosphere. Cells were broken by passage through a French™ pressure cell press (Thermo IEC) under a constant nitrogen gas stream. Cell membranes were removed by ultra-centrifugation at 98 k×*g* for 60 min at 4 °C. All subsequent purification steps were carried out in an anaerobic glove box (Belle Technologies, O_2 < 1 ppm). Soluble crude extract was batched with 10 mL anaerobic StrepTactin Sepharose (IBA) resin resuspended in buffer A for 4 h at 4 °C. The resin was added to a small Econo-Pac disposable chromatography column (BioRad) and then washed with 30 mL buffer A by gravity flow to remove unbound proteins. TF-PceA was eluted from the column with 40 mL buffer B (buffer A plus 2.5 mM D-desthiobiotin). Fractions were analyzed by SDS–PAGE and protein concentrations calculated by the Bradford method with bovine serum albumin (BSA) as a standard [25]. Elution fractions containing pure TF-PceA were combined and anaerobically concentrated to ~10 μM using a 100 kDa molecular weight cut-off (MWCO) spin concentrator. Anaerobic conditions were maintained by sealing the spin concentrator in the glove box. Proteolytic cleavage of TF-PceA was performed with HRV 3C protease (Novagen) in buffer B plus 0.1% (w/v) n-octyl-β-D-glucopyranoside (BOG)[1] for 16–24 h at 4 °C in an anaerobic sealed tube with gentle shaking.

Reconstitution of 4Fe-4S clusters and cobalamin uptake

All experiments with TF-PceA were carried out in anaerobic 50 mM Hepes pH 8.0, 150 mM NaCl and 10% glycerol, unless otherwise stated. Chemical reconstitution of the PceA 4Fe-4S clusters was performed according to [26]. Briefly, 10 μM of TF-PceA was fully reduced in the glove box at 21 °C with 2% β-mercaptoethanol for 1 h under strictly anaerobic conditions. Na_2S and $FeCl_3$ (Sigma) were slowly added to a final concentration of 150 μM each. The reconstitution was carried out for 16 h at 4 °C with gentle shaking, in either the presence or absence of HRV 3C protease. Methylcobalamin (Sigma) was incorporated into PceA during the limited proteolysis with HRV 3C. Excess cobalamin was removed from the sample by dialysis of the sample into 500 fold excess of anaerobic buffer. The dialysis step was performed in parallel to the cobalamin uptake step.

UV–Vis and EPR measurements

UV–Vis absorbance spectra were recorded with a Cary UV–Vis spectrophotometer situated inside an anaerobic glove box. All spectra recorded were baseline corrected with buffer or the collected flow through. Reducing conditions were achieved by the addition of 5 mM Na-dithionite. EPR samples for cobalamin incorporation by PceA were prepared by further concentrating the protein sample from the dialysis tube with a 30 kDa cut-off spin

[1] *Abbreviations used:* BOG, n-octyl-β-D-glucopyranoside; *cspA*, cold shock promoter A; EPR, electron paramagnetic resonance; HRV 3C, human rhinovirus 3C protease; IPTG, isopropyl-β-D-thiogalactopyranoside; K, temperature in Kelvin; MBP, maltose binding protein; MWCO, molecular weight cut-off; NMR, nuclear magnetic resonance; PCE, tetrachloroethene; PCR, polymerase chain reaction; RdhA, reductive dehalogenase; SUMO, small ubiquitin-like modifier; TAT, twin arginine translocation; TCE, trichloroethene; TF, trigger factor; TF-PceA, trigger factor – PceA fusion protein.