Trace Nutrients Research 28 : 58－64 (2011)

Original Article

Function of $FADH_2$-dependent 2-haloacrylate hydratase from a 2-chloroacrylate-utilizing bacterium, *Burkholderia* sp. WS

Anoop VASUDEVAN[1)], Michiyo FUJITA[1)], Atsushi KURATA[1, 2)],
Jun KAWAMOTO[1)], Nobuyoshi ESAKI[1)] and Tatsuo KURIHARA[1)]
[1)]*Institute for Chemical Research, Kyoto University**,
[2)]*Graduate School of Applied Biological Chemistry, Kinki University***

Summary

Enzymes that catalyze the degradation of organohalogen compounds are useful in environmental technology and chemical industry. *Burkholderia* sp. WS is a Gram-negative bacterium that can utilize an aliphatic unsaturated organohalogen compound, 2-chloroacrylate (2-CAA), as its sole carbon source. The production of 2 proteins, CAA43 and CAA67_WS, is induced when the bacterium is grown in 2-CAA medium. CAA43 catalyzes the conversion of 2-CAA into (*S*)-2-chloropropionate, whereas the function of CAA67_WS remains unknown. Recently, a homolog of CAA67_WS from *Pseudomonas* sp. YL (CAA67_YL), subsequently named 2-haloacrylate hydratase, was shown to catalyze the $FADH_2$-dependent hydration of 2-CAA to produce pyruvate. Our results suggest that CAA67_WS has a similar activity. Gene-disruption studies of CAA43 and CAA67_WS indicated that CAA67_WS is physiologically more important in the assimilation of 2-CAA in *Burkholderia* sp. WS. The enzyme CAA67_WS was purified from *Burkholderia* sp. WS and characterized. The UV-visible spectrum of the protein indicated the presence of bound flavin. CAA67_WS released chloride ions from 2-CAA in the presence of FAD and reducing agents such as NAD(P)H. CAA67_WS is similar to CAA67_YL in these respects. However, while the reduced form of flavin mononucleotide (FMN) served as a cofactor for CAA67_WS, it did not for CAA67_YL. CAA67_YL is a bifunctional enzyme that catalyzes the hydration of 2-CAA and the reduction of FAD by using NADH; CAA67_WS did not catalyze the reduction of FAD. Thus, comparative studies of these 2 proteins can provide valuable information on the structure-function relationship of these proteins.

Abbreviations: 2-CAA, 2-chloroacrylic acid
(*S*)-2-CPA, (*S*)-2-chloropropionic acid
ESI-MS, electrospray ionization mass spectrometry

Introduction

Halogenated organic compounds are one of the largest groups of environmental pollutants because of their widespread use in the past as herbicides, fungicides, solvents, plasticizers, and intermediates for chemical synthesis[1)]. Apart from the organohalogen compounds produced industrially, many are produced biologically or by natural abiogenic processes such as volcanic eruption[2)]. Many organohalogen compounds are hazardous to the environment due to their toxicity, bioconcentration, and long lifespans. Many microorganisms and enzymes capable of degrading these compounds have been reported[3-5)]. Removal of the halogen atom from these organohalogen compounds is a major way of detoxifying these compounds, another being conversion of these compounds to other organohalogen compounds that can be easily metabolized.

The enzymes that catalyze the removal of halogen atoms from organohalogen compounds are called dehalogenases. Some of the dehalogenases characterized so far can act on unsaturated aliphatic organohalogen compounds such as *cis/trans*-3-chloroacrylic acid and 2-chloromaleylacetate[6-8)]. Previously, 3 bacterial strains were isolated from garden soil in Uji, Kyoto, Japan; these strains can assimilate 2-chloroacrylic acid (2-CAA) as a sole carbon source. These strains were identified as *Pseudomonas* sp. YL, *Burkholderia* sp. WS (formerly *Pseudomonas* sp. WS), and *Burkholderia* sp. WL (formerly *Pseudomonas* sp. WL)[9)]. 2-CAA is a bacterial metabolite of 2-chloroallyl alcohol, which is an intermediate or byproduct in industrial herbicide synthe-

* Address : Uji, Kyoto 611-0011, Japan
** Address : 3327-204 Nakamachi, Nara 631-8505, Japan

sis[10]. Rats excrete 2-CAA when treated orally with herbicides containing a haloallyl substituent[11].

Two inducibly produced proteins, CAA43 and CAA67_WS, were expressed when *Burkholderia* sp. WS was grown in 2-CAA medium[12]. The protein CAA43 was found to be 2-haloacrylate reductase. It catalyzes the reduction of 2-CAA to (*S*)-2-chloropropionic acid ((*S*)-2-CPA), using NADPH as a co-substrate (Fig. 1). The function of the other protein, CAA67_WS, could not be determined earlier due to the difficulty in construction of an effective recombinant expression system. It was found that *Pseudomonas* sp. YL produces a homolog of CAA67_WS (sequence identity, 84.6%) when it grows on 2-CAA. This enzyme was recently characterized and found to be 2-haloacrylate hydratase, which catalyzes the hydration of 2-CAA to produce pyruvate[13]. The present study analyzes the function of CAA67_WS in *Burkholderia* sp. WS and its physiological importance in metabolism of 2-CAA.

Materials and Methods

Materials

2-CAA was purchased from Lancaster Synthesis Ltd. (Lancashire, UK). All other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan). Restriction enzymes and kits for genetic manipulation were purchased from Takara Bio (Otsu, Japan), Toyobo (Osaka, Japan), and Qiagen Ltd. (West Sussex, UK).

Generation of gene-disrupted mutants

The gene fragments corresponding to 5′- and 3′-halves of *caa67_ws* and *caa43* (GenBank accession number: AB196962) were amplified from the genomic DNA of *Burkholderia* sp. WS using the following primers: (1) sense primer (5′-AACTGCAGATGGTAATGGCAGCGGTAATTCATAAGAAG-3′) and antisense primer (5′-TATCGATGATAAGCTGTCACGGCCCAAGGAACCATGATGTG-3′) and sense primer (5′-AACGGATTCACCACTCCAAGAACGCCACCTCGGCGCTACC-3′) and antisense primer (5′-GCTCTAGACTACGCTTGCGGAAGCAAAACAATCGAGCC-3′) for the 5′- and 3′-halves of the *caa43* gene, respectively; and (2) sense primer (5′-AACTGCAGATGTCGGATGTTCTTGTAACAGACGTGTTG-3′) and antisense primer (5′-TTATCGATGATAAGCTGTCATTCGGCCCCCGTCGTCAATG-3′) and sense primer (5′-AACGGATTCACCACTCCAAGAAACGTCCTGGTGAAGCATC-3′) and antisense primer (5′-GCTCTAGATTAGAGGGGAACGTCTTTGAAATGCAACGC-3′) for the 5′- and 3′-halves of the *caa67_ws* gene, respectively. The gene for tetracycline-resistance (*tet*) from the plasmid pBR322 was amplified using sense primer (5′-CACATCATGGTTCCTTGGGCGTGACAGCTTATCATCGATA-3′) and antisense primer (5′-GGTAGCGCCGAGGTGGCGTTCTTGGAGTGGTGAATCCGTT-3′) for inserting into *caa43,* and the sense primer (5′-TCATTGACGACGGGGGCCGAATGACAGCTTATCATCGATA-3′) and antisense primer (5′-GATGCTTCACCAGGACGTTTCTTGGAGTGGTGAATCCGTT-3′) for inserting into *caa67_ws*. These were then used in the construction of plasmids for disruption of *caa43* and *caa67_ws*. The *tet* gene was placed in between the 5′- and 3′-halves of these genes by overlap extension PCR. The resultant fragments were digested with restriction enzymes PstI and XbaI for inserting into pK18*mobsacB* plasmid. These plasmids were introduced into competent *Burkholderia* sp. WS cells, either by electroporation or by conjugation with *Escherichia coli* S17-1. The mutants *Δcaa43* and *Δcaa67_ws* were verified by Southern blot hybridization.

Culture conditions

The wild-type *Burkholderia* sp. WS, *Δcaa43*, and *Δcaa67_ws* were grown aerobically at 28℃ in a medium containing 0.2% $(NH_4)_2SO_4$, 0.05% Bacto yeast extract (Difco), 0.1% K_2HPO_4, 0.1% NaH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$ (pH 7.1), and various carbon sources. Either 0.2% 2-CAA, lactate, (*S*)-2-CPA, or both 2-CAA and (*S*)-2-CPA together were added to the medium as a carbon source.

Purification of native CAA67_WS

The cells of *Burkholderia* sp. WS were harvested at the late logarithmic phase, washed twice with 50 mM potassium phosphate buffer (pH 7.1) containing 1 mM dithiothreitol, resuspended in the same buffer, and disrupted by passing through a French press 3 times at 1000 psi. Cell debris was removed by centrifugation. The cell-free extract thus obtained was subjected to ammonium sulfate precipitation at 40% saturation. The precipitated proteins were removed by centrifugation, and the supernatant was diluted to 30% ammonium sulfate saturation. This supernatant that contained 2-haloacrylate hydratase activity was then applied to a Toyopearl Butyl 650 M column pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.1) containing 1 mM dithiothreitol and 30% ammonium sulfate. The unbound proteins were removed by washing with the same buffer. CAA67_WS was then eluted using a linear gradient of 30–0% ammonium sulfate. The active fractions were collected, concentrated, and dialyzed against 5 mM potassium phosphate buffer (pH 7.1) containing 1 mM dithiothreitol. This was then applied to a Toyo-

pearl DEAE 650 M column pre-equilibrated with 5 mM potassium phosphate buffer (pH 7.1) containing 1 mM dithiothreitol. After washing the column to remove unbound proteins, CAA67_WS was eluted with a linear gradient of 5–60 mM potassium phosphate buffer (pH 7.1) containing 1 mM dithiothreitol. The active fractions were pooled and concentrated using a Millipore Amicon 30-kDa membrane filter. These fractions were then subjected to gel filtration using a HiLoad 16/60 Superdex 200 pg column and 50 mM sodium phosphate buffer (pH 7.1) containing 150 mM sodium sulfate. The active fractions were pooled, concentrated to 5.3 mg/ml, and stored at −80℃ until use.

Enzyme assay

The enzymatic activity of CAA67_WS was determined under anaerobic conditions by measuring the amount of chloride ions released from 2-CAA, according to the method described by Iwasaki *et al*[18]. The reaction mixture (100 μl) contained 60 mM Tris sulfate buffer (pH 9.0), 3.5 mM 2-CAA neutralized with an equimolar amount of NaOH, 0.1 mM FAD or FMN, and 10 mM NAD(P)H. The reaction was carried out at 30℃ and terminated by the addition of 11.1 μl of 1.5 M sulfuric acid.

Analysis of 2-CAA degradation product by electrospray ionization mass spectrometry (ESI-MS)

A 500-μl reaction mixture containing 60 mM ammonium acetate buffer (pH 7.1), 5 mM 2-CAA, 20 mM NAD(P)H, 0.1 mM FAD or FMN, and 10% (v/v) cell-free extract was incubated at 28℃ for 24 h. The reaction was terminated by the addition of 1 ml acetonitrile. The reaction mixture was centrifuged, filtered, and diluted with acetonitrile/10 mM ammonium acetate (1:1). After dilution, the mixture was analyzed by ESI-MS using an API3000 LC/MS/MS system (Applied Biosystems, Foster City, CA) in the negative-ion mode.

UV-visible absorbance spectrometry

The reduction of CAA67_WS-bound FAD was monitored anaerobically with a UV-visible spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan) for which the cell holder was installed inside the anaerobic chamber. The reaction mixture (500 μl) contained 1 mM NAD(P)H, 0.05 mM FAD, 60 mM Tris sulfate buffer (pH 9.0), and 20 μl purified enzyme. The analysis was done at room temperature.

Molecular weight determination

The subunit molecular weight of CAA67_WS was determined by SDS-PAGE. The molecular weight of the native enzyme was analyzed by gel filtration with an ÄKTA Explorer 10S system (GE Healthcare UK Ltd., Buckinghamshire, United Kingdom) equipped with a HiLoad 16/60 Superdex 200 pg column (GE Healthcare UK Ltd.). Molecular mass marker proteins (Oriental Yeast Co. Ltd., Tokyo, Japan) consisting of glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (32 kDa), and cytochrome c (12.4 kDa) were used as standards.

Results and Discussion

This study describes the biochemical characteristics and physiological importance of 2-haloacrylate hydratase expressed in *Burkholderia* sp. WS grown in 2-CAA medium. As reported previously[12], CAA67_WS and CAA43 are able to be inducibly produced when *Burkholderia* sp. WS is grown in 2-CAA medium. The protein CAA43 was subsequently characterized as 2-haloacrylate reductase, which catalyzes the conversion of 2-CAA into (*S*)-2-CPA in an NADPH-dependent manner (Fig. 1).

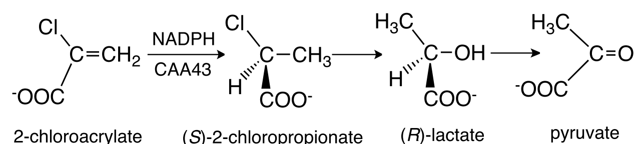

Fig. 1 Proposed pathway for the degradation of 2-CAA by 2-haloacrylate reductase (CAA43) in *Burkholderia* sp. WS.

Based on the amino acid sequences of CAA67_WS and CAA43, degenerate primers were designed, and a part of the gene cluster containing these 2 proteins was amplified by inverse PCR. The genes coding for CAA67_WS and CAA43 were found to be located next to each other on the genome of *Burkholderia* sp. WS[12] (GenBank accession number: AB196962).

The gene encoding CAA67_WS was present immediately upstream of CAA43, at a distance of 267 bp. Putative Shine-Dalgarno sequences, AGGAGG and AGGAG, were found in the upstream regions of the initiation codons of the CAA67_WS gene and the CAA43 gene, respectively, but −35 (CTTGATGT) and −10 (TTTAAT) sequences were found only in the upstream region of the CAA67_WS gene. In addition, the inducible synthesis of both CAA67_WS and CAA43 when the cells are grown in 2-CAA medium suggests that these genes are present in the same operon.

Primary structure analysis of CAA67_WS showed that it shares sequence similarity with FAD-dependent enzymes L-aspartate oxidase from *Escherichia coli*[14] (NCBI accession number 5542180; 17.6% identity) and fumarate reductase (subunit A) from *Wolinella succinogenes*[15] (NCBI

accession number 37538290; 17.2% identity). An FAD-binding motif (GXGXXG) was also observed in the region 13 to 18 of the amino acid sequence of CAA67_WS. These observations suggest that CAA67_WS is a FAD-dependent enzyme and probably an oxidoreductase functioning in the metabolic pathway of 2-CAA. However, since 2-haloacrylate reductase (CAA43) has been described as an enzyme that directly acts on 2-CAA, we initially speculated that CAA67_WS plays some supportive function to that of CAA43, such as generation of NADPH[12].

In order to understand the physiological role of CAA67_WS, the mutants *Δcaa43* and *Δcaa67_ws* were created by disrupting the genes for CAA43 and CAA67_WS, respectively, as described in the "Materials and Methods." The growth characteristics of these strains in 2-CAA, (*S*)-2-CPA, and lactate media were examined and compared with that of the wild-type strain. (*S*)-2-CPA and lactate were used as both are supposed to be metabolites of 2-CAA (as shown in Fig. 1). No significant change in the growth profile was observed when the 3 strains were grown on lactate as the carbon source (Fig. 2B). In the medium containing (*S*)-2-CPA, both the mutant strains showed a poorer growth than the wild-type strain, but the overall growth of all the 3 strains was much lower than that in lactate medium (Fig. 2A). When grown in the 2-CAA medium, the *Δcaa43* strain did not show growth retardation compared with the wild-type strain (Fig. 2C). In contrast, 2-CAA did not support the growth of the *Δcaa67_ws* strain. This indicated that there is a CAA43-independent 2-CAA metabolic pathway, in which CAA67_WS plays a major role. The growth characteristics of the different strains suggest that this second pathway is physiologically more important in *Burkholderia* sp. WS, enabling this strain to utilize 2-CAA as a sole carbon source.

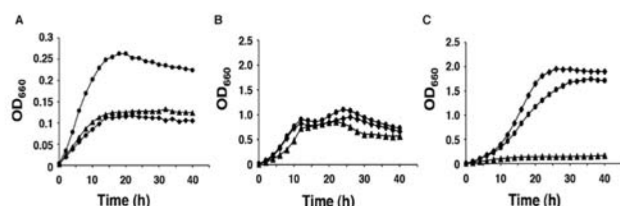

Fig. 2 Growth of the wild-type, *Δcaa43*, and *Δcaa67* strains of *Burkholderia* sp. WS on various carbon sources. The carbon sources examined were (*S*)-2-CPA (A), lactate (B), and 2-CAA (C). Wild-type strain (closed circle), *Δcaa43* strain (closed diamond), and *Δcaa67* strain (closed triangle).

To estimate the function of CAA67_WS, 2-CAA degradation activities of the cell-free extracts of the wild-type and *Δcaa43* strains were analyzed by ESI-MS. Under aerobic conditions, using the wild-type strain extract in the presence of NADPH, the peak corresponding to [^{35}Cl]-2-CAA at $m/z = 105$ decreased, while the peaks corresponding to [^{37}Cl]-(*S*)-2-CPA ($m/z = 109$) and lactate ($m/z = 89$) increased in a time-dependent manner (data not shown). This was expected due to the action of 2-haloacrylate reductase (CAA43). In contrast, the reaction did not proceed when the cell-free extract from the *Δcaa43* strain was used as 2-haloacrylate reductase is not expressed. When the reaction was carried out under anaerobic conditions and in the presence of NADPH and FAD, the cell-free extract of the wild-type strain converted 2-CAA into (*S*)-2-CPA, lactate, and pyruvate ($m/z = 87$) (Fig. 3A). Under the same conditions, peaks corresponding to lactate and pyru-

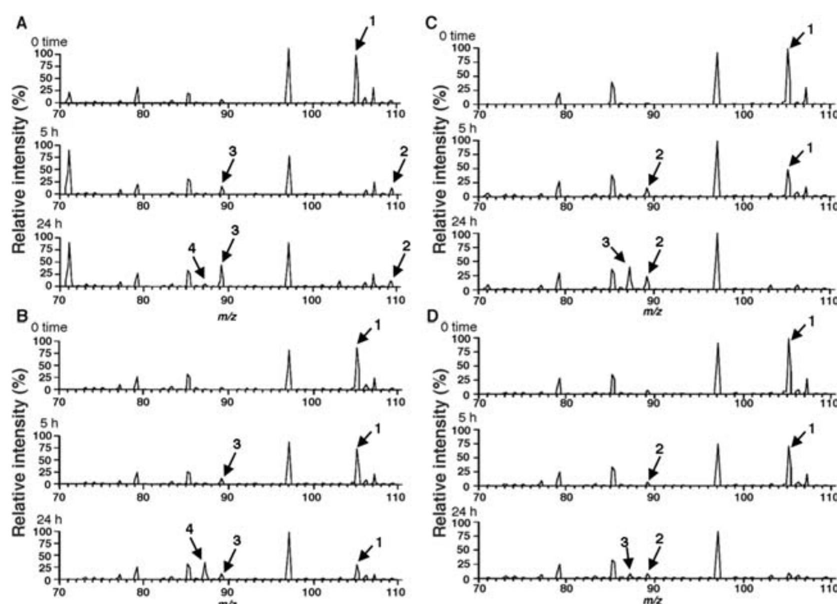

Fig. 3 Mass spectrometric monitoring of 2-CAA degradation in the presence of NAD(P)H and FAD under anaerobic conditions. Cell-free extracts of the wild-type strain (A and C) and the *Δcaa43* strain (B and D) were used. NADPH was added for A and B, whereas NADH was added for C and D. In A and B, arrows indicate 2-CAA (1), (*S*)-2-CPA (2), lactate (3), and pyruvate (4). In C and D, arrows indicate 2-CAA (1), lactate (2), and pyruvate (3). The peak at *m/z* value of 85.0 is of methacrylate, an internal standard.

vate were detected when the cell-free extract from the Δ*caa43* strain was used (Fig. 3B). However, (*S*)-2-CPA was not produced because of the absence of 2-haloacrylate reductase (CAA43), which catalyzes the conversion of 2-CAA into (*S*)-2-CPA. When NADH was used instead of NADPH in the above condition, cell-free extracts from both the strains converted 2-CAA into lactate and pyruvate (Fig. 3C and 3D). These results confirm the presence of a CAA43-independent pathway for 2-CAA metabolism. It can also be inferred that in this pathway, 2-CAA is converted directly into lactate or pyruvate, without the generation of (*S*)-2-CPA.

Recently, a homolog of CAA67_WS, CAA67_YL, from the bacterium *Pseudomonas* sp. YL was characterized as an $FADH_2$-dependent 2-haloacrylate hydratase that catalyzes the conversion of 2-CAA into pyruvate (Fig. 4)[13]. CAA67_YL shares 84.6% sequence identity with CAA67_WS from *Burkholderia* sp. WS. Thus, it is highly likely that CAA67_WS from *Burkholderia* sp. WS catalyzes the same reaction as CAA67_YL.

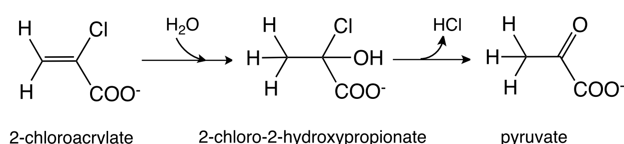

Fig. 4 Reaction scheme of conversion of 2-CAA by CAA67_YL from *Pseudomonas* sp. YL.

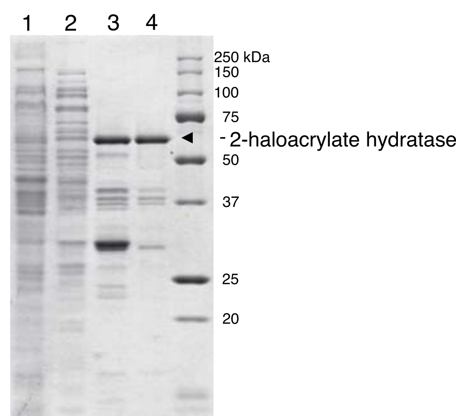

Fig. 5 SDS-PAGE analysis of samples containing CAA67_WS at different purification stages. Crude extract (1), Butyl-Toyopearl (2), DEAE-Toyopearl (3), and Superdex 200 pg (4).

The earlier attempts for recombinant expression of CAA67_WS in *E.coli* were not successful due to complications in solubilizing and refolding the protein following its production in inclusion bodies. Therefore, in this study, the enzyme was purified from a culture of the wild-type strain of *Burkholderia* sp. WS grown in a medium containing 0.2% 2-CAA and 0.2% 2-CPA (Table 1 and Fig. 5), as described in the "Materials and Methods."

The molecular mass of the purified CAA67_WS was approximately 60,000 Da as measured by SDS-PAGE, which agrees with the theoretical value of 58,684 Da deduced from its primary structure. The molecular mass determined by gel filtration was 48,500 Da, suggesting that the enzyme is monomeric. Purified CAA67_WS showed 2-haloacrylate hydratase activity, as expected from its structural similarity to CAA67_YL. The specific activity of the purified enzyme was 60 mU/mg.

Purified CAA67_WS contained an oxidized form of FAD, as judged by its absorption spectrum. The molar ratio of FAD to the protein was approximately 0.15. This ratio increased to approximately 0.45 after incubation with an excess amount of externally added FAD, suggesting that at least about 50% of the purified enzyme was irreversibly inactivated during the purification process.

The requirement of cofactors was further tested by analyzing the enzymatic activity under various conditions (Fig. 6A). When the purified protein was incubated with 2-CAA in the presence of 0.1 mM FAD and 10 mM NADH under anaerobic conditions, 2-CAA was degraded, and chloride ions were liberated. The enzyme activity, as ob-

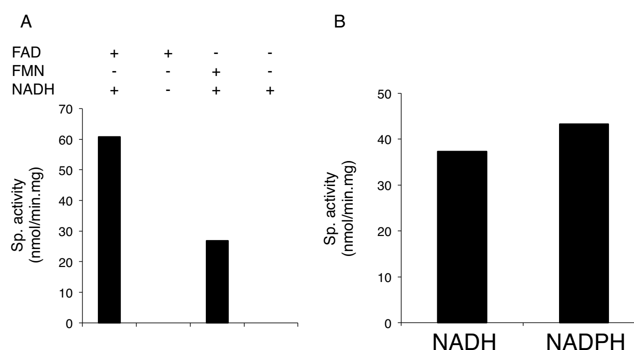

Fig. 6 Cofactor requirement of CAA67_WS. Enzymatic reactions were carried out under anaerobic conditions.

Table 1. Purification of CAA67_WS from *Burkholderia* sp. WS cells[a]

Purification step	Total activity (U)	Total protein (mg)	Specific activity (mU/mg)	Yield (%)	Purification (fold)
Crude extract	7.2	750	9.6	100	1
Butyl-Toyopearl	1.4	120	12	19	1.3
DEAE-Toyopearl	0.13	4.9	27	1.8	2.8
Superdex 200 pg	0.029	0.48	60	0.40	6.3

[a] The enzyme activities were determined by measuring halide ions released from 2-CAA

served by the release of chloride ions, decreased to 44% when FAD was replaced by FMN. No enzyme activity could be detected when the reaction was performed without NADH. The reaction in the presence of NADH, but in the absence of FAD or FMN, also did not result in the release of chloride ions. This clearly shows that reduced FAD ($FADH_2$) is the cofactor of CAA67_WS. The results also indicate that reduced FMN ($FMNH_2$) serves as a cofactor, although less efficiently than $FADH_2$. This is in clear contrast with CAA67_YL, for which $FMNH_2$ does not serve as a cofactor. The requirement of NADH showed that the presence of a reducing agent is essential to maintain the reduced form of FAD. The reaction proceeded when NADH was replaced with NADPH (Fig. 6B) or sodium dithionite (data not shown).

The reduction of FAD into its reduced form, $FADH_2$, by NAD(P)H can either be a chemical process or an enzymatic one. In order to understand this, the spectrum of FAD was analyzed at various time intervals in the presence of 1 mM NAD(P)H, with or without CAA67_WS (Fig. 7). A decrease in the absorption spectra at 450 nm indicates the conversion of FAD into $FADH_2$. The decrease in spectra observed was similar both in the presence and absence of CAA67_WS, irrespective of whether NADH or NADPH was used. Hence, it can be considered that FAD is reduced to $FADH_2$ non-enzymatically by CAA67_WS, unlike CAA67_YL, which catalyzes the NADH-dependent reduction of FAD in addition to hydration of 2-CAA[16].

From the results presented in this study, it can be concluded that 2-haloacrylate hydratase plays an important role in the metabolism of 2-CAA in *Burkholderia* sp. WS and is essential for the survival of the bacterium in a medium with 2-CAA as the sole carbon source. 2-Haloacrylate hydratase is inducibly produced when *Burkholderia* sp. WS is cultured in a medium containing 2-CAA. The enzyme uses $FADH_2$ and $FMNH_2$ as its cofactor and is not biologically active if the flavin is not in its reduced form. Therefore, the redox environment in the vicinity of the enzyme is critical. It could be assumed that the enzyme catalyzes the hydration of 2-CAA to produce 2-chloro-2-hydroxypropionic acid, which is chemically unstable and spontaneously dechlorinated to pyruvate, releasing HCl. Dehalogenation through hydration has been reported for some unsaturated aliphatic compounds like *cis*- and *trans*-3-chloroacrylate[6, 7] and aromatic organohalogens like chlorothalonil[17]. 2-Haloacrylate hydratase is different from the

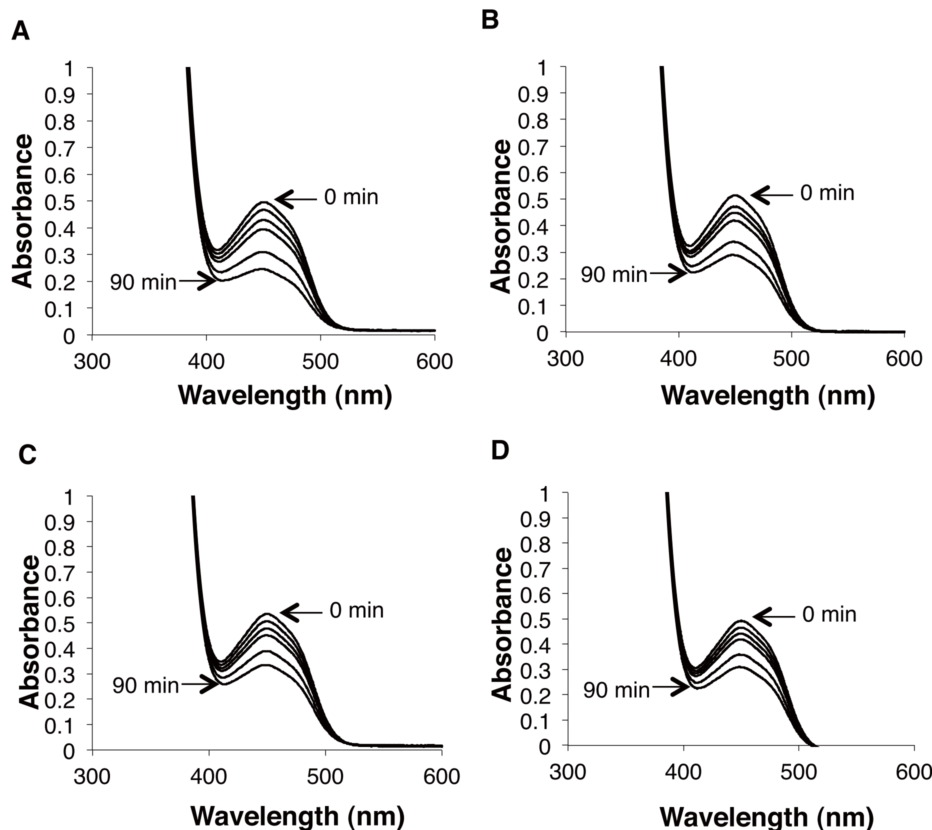

Fig. 7 Changes in the UV-visible spectrum of FAD bound to CAA67_WS. Incubation was carried out as described in Materials and Methods in Tris sulfate buffer pH 9.0 with 0.05 mM FAD. (A) 1 mM NADH was added in the presence of 2.3 μM enzyme. (B) 1 mM NADH was added in the absence of enzyme. (C) 1 mM NADPH was added in the presence of 2.3 μM enzyme. (D) 1 mM NADPH was added in the absence of enzyme. The changes in the spectrum were recorded at 0, 10, 20, 30, 60, and 90 minutes, displayed from top to bottom, respectively.

enzymes for dehalogenation of 3-chloroacrylate in its absolute requirement for reduced flavin as a cofactor. Further mechanistic and genetic analyses are required to explain this mechanism more clearly. CAA67_WS and CAA67_YL are similar to each other in their ability to catalyze the dechlorination of 2-CAA in a reduced flavin-dependent manner. However, $FMNH_2$ serves as a cofactor only for CAA67_WS, and NADH-dependent FAD reductase activity is found only for CAA67_YL. Thus, comparative studies of these enzymes would give us valuable information regarding the structure-function relationship of 2-haloacrylate hydratase. The information would contribute to the development of enzymes that are useful in chemical industry and bioremediation of environments polluted by organohalogen compounds.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (B) (17404021) from JSPS (to T. K.), a grant for Research for Promoting Technological Seeds from JST (to T. K.), and the Collaborative Research Program of Institute for Chemical Research, Kyoto University (grant #2010-53 and 2011-50).

References

1) Fetzner S, Lingens F (1994) Bacterial dehalogenases: biochemistry, genetics, and biotechnological applications. Microbial Rev 58: 641-685.

2) Gribble GW (2003) The diversity of naturally produced organohalogens. Chemosphere 52: 289-297.

3) Janssen DB, Dinkla IJ, Poelarends GJ, Terpstra P (2005) Bacterial degradation of xenobiotic compounds: evolution and distribution of novel enzyme activities. Environ. Microbiol. 7: 1868-1882.

4) Kurihara T, Esaki N (2008) Bacterial hydrolytic dehalogenases related enzymes: occurrences, reaction mechanisms and applications. Chem. Rec. 8: 67-74.

5) Swanson PE (1999) Dehalogenases applied to industrial-scale biocatalysis. Curr. Opin. Biotechnol. 10: 365-369.

6) de Jong RM, Bazzaco P, Poelarends GJ, Johnson Jr. WH, Kim YJ, Burks EA, Serrano H, Thunnissen AM, Whitman CP, Dijkstra BW (2007) Crystal structures of native and inactivated *cis*-3-chloroacrylic acid dehalogenase. Structural basis for substrate specificity and inactivation by (*R*)-oxirane-2-carboxylate. J. Biol. Chem. 282: 2440-2449.

7) Poelarends GJ, Whitman CP (2004) Evolution of enzymatic activity in the tautomerase superfamily: mechanistic and structural studies of the 1,3-dichloropropene catabolic enzymes. Bioorg. Chem. 32: 376-392.

8) Endo R, Kamakura M, Miyauchi K, Fukuda M, Ohtsubo Y, Tsuda M, Nagata Y (2005) Identification and characterization of genes involved in the downstream degradation pathway of γ-hexachlorocyclohexane in *Sphingomonas paucimobilis* UT26. J. Bacteriol. 187: 847-853.

9) Hasan AKMQ, Takada H, Koshikawa H, Liu JQ, Kurihara T, Esaki N, Soda K (1994) Two kinds of 2-haloacid dehalogenases from *Pseudomonas* sp. YL induced by 2-chloroacrylate and 2-chloropropionate. Biosci. Biotechnol. Biochem. 58: 1599-1602.

10) van der Waarde JJ, Kok R, Janssen DB (1993) Degradation of 2-chloroallyl alcohol by a *Pseudomonas* sp. Appl. Environ. Microbiol. 59: 528-535.

11) Marsden PJ, Casida JE (1982) 2-Haloacrylic acids as indicators of mutagenic 2-haloacrolein intermediates in mammalian metabolism of selected promutagens and carcinogens. J. Agric. Food. Chem. 30: 627-631.

12) Kurata A, Kurihara T, Kamachi H, Esaki N (2005) 2-Haloacrylate reductase, a novel enzyme of the medium chain dehydrogenase/reductase superfamily that catalyzes the reduction of a carbon-carbon double bond of unsaturated organohalogen compounds. J. Biol. Chem. 280: 20286-20291.

13) Mowafy AM, Kurihara T, Kurata A, Uemura T, Esaki N (2010) 2-Haloacrylate hydratase, a new class of flavoenzyme that catalyzes the addition of water to the substrate for dehalogenation. Appl. Environ. Microbiol. 76: 6032-6037.

14) Bossi RT, Negri A, Tedeschi G, Mattevi A (2002) Structure of FAD-bound L-aspartate oxidase: Insight into substrate specificity and catalysis. Biochemistry 41: 3018-3024.

15) Lancaster CRD, Kroger A, Auer M, Michel H (1999) Structure of fumarate reductase from *Wolinella succinogenes* at 2.2 Å resolution. Nature 402: 377-385.

16) Mowafy AM, Kurihara T, Esaki N (2010) 2-Haloacrylate hydratase is a bifunctional enzyme with NADH-dependent FAD reductase activity. Trace Nutrients Research 27: 52-55.

17) Wang G, Li R, Li S, Jiang J (2010) A novel hydrolytic dehalogenase for the chlorinated aromatic compound chlorothalonil. J. Bacteriol. 192: 2737-2745.

18) Iwasaki I, Utsumi S, Hagino K, Ozawa T (1956) A new spectrophotometric method for the determination of small amounts of chloride using the mercuric thiocyanate method. Bull. Chem. Soc. Jpn. 29: 860-864.