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Original Article

2-Haloacrylate Hydratase Is a Bifunctional Enzyme with NADH-dependent FAD Reductase Activity

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Summary

2-Haloacrylate hydratase from *Pseudomonas* sp. YL catalyzes the hydration of 2-chloroacrylate to produce pyruvate and HCl. Although the conversion does not involve a net change in the redox state, the enzyme requires reduced FAD, which is not consumed during substrate turnover. It was not clear how the reduced form of FAD is generated *in vivo*. The examination of the primary structure of the enzyme has revealed the presence of an unusual nucleotide-binding fingerprint motif consisting of the sequence AXXGXXG; this finding suggests that the enzyme may bind to NADH or NADPH. The UV-visible spectroscopic analyses indicated that 2-haloacrylate hydratase catalyzes the reduction of FAD at the expense of NADH. Thus, 2-haloacrylate hydratase is a bifunctional enzyme with both NADH-dependent FAD reductase activity and $FADH_2$-dependent 2-haloacrylate hydratase activity.

Abbreviations: 2-CAA, 2-chloroacrylic acid, DTT, dithiothreitol

Introduction

The carbon-halogen bond can be cleaved by specific enzymes called dehalogenases[1]. There is a broad range of dehalogenases. These can be classified into different protein superfamilies and have fundamentally different catalytic mechanisms[2]. Little information is available on the metabolism of unsaturated organohalogens as compared to that on the saturated counterparts. Previously, three bacterial strains were isolated for their ability to grow on 2-chloroacrylate (2-CAA)[3]. In *Burkholderia* sp. WS, two different proteins are inducibly expressed in the presence of 2-CAA[4]). We previously reported that one of those proteins, i.e., CAA43, catalyzes the reduction of 2-CAA to produce L-2-chloropropionate by using NADPH as a cosubstrate, and we named this enzyme 2-haloacrylate reductase. However, the function of the second protein, i.e., CAA67_WS, was unclear until recently when its homologue (CAA67_YL, 84.6% identity) from *Pseudomonas* sp. YL was characterized[5]. CAA67_YL, named 2-haloacrylate hydratase, catalyzes the hydration of 2-CAA to produce pyruvate. Although this conversion does not involve an overall oxidation or reduction reaction, this new enzyme requires reduced FAD for catalysis. The reducing agent was not stoichiometrically consumed during this reaction. This suggests that $FADH_2$ is conserved in the catalytic cycle because of regeneration[5]. The possible role of the reduced flavin in the hydration reaction catalyzed by CAA67_YL has been investigated and the results led us to propose that the reduced form of FAD acts as a radical catalyst providing one electron to 2-CAA in order to facilitate hydration (Mowafy *et al.*, unpublished data). One of the major unanswered questions about this enzyme is how $FADH_2$, which is required for the reaction, is produced. Although $FADH_2$ is regenerated in the catalytic cycle under anaerobic conditions, it may be oxidized and thus may become catalytically nonfunctional under aerobic conditions where this bacterium grows. In the present study, we analyzed the mechanism of $FADH_2$ formation and found that CAA67_YL catalyzes the reduction of FAD at the expense of NADH.

Materials and Methods

1. Purification of the recombinant CAA67_YL

Expression of CAA67_YL in *Escherichia coli* BL21(DE3) was carried out using pET-21a(+) as the expression vector as described in a previous report[5]. After elution through a DEAE-Toyopearl 650M column and dialysis against the standard buffer, i.e., a 60 mM KPB (pH 7.1) containing

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1 mM dithiothreitol (DTT), the enzyme solution was treated with 40% ammonium sulfate. After centrifugation, the supernatant was applied on a Butyl-Toyopearl 650M column pre-equilibrated with the standard buffer containing 40% ammonium sulfate, and the unbound proteins were removed by washing with the same buffer. CAA67_YL was then eluted by using a linear gradient of the standard buffer containing 40-0% ammonium sulfate. The active fractions were collected, dialyzed against the standard buffer, concentrated to 54 mg/mL, and stored at −80°C until use. The protein concentration was determined by the method of Bradford[6] using bovine serum albumin as the standard.

2. Enzyme assay

The hydratase activity of CAA67_YL was measured under anaerobic conditions by measuring the amount of chloride ions released, according to the method described by Iwasaki *et al.*[7]. The reaction mixture (100 μL) contained a 60 mM Tris sulfate buffer (pH 9.0), 3.5 mM 2-CAA neutralized with an equimolar amount of NaOH, 0.1 mM FAD, and 10 mM NADH. The reaction was carried out at 35°C for 1–5 min and terminated by the addition of 11.1 μL of 1.5 M sulfuric acid.

3. UV-visible absorbance spectroscopy

The reduction of CAA67_YL-bound FAD was monitored anaerobically with a UV-visible spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan) in which the cell is installed inside the glove box. All assays were carried out in a 60 mM Tris sulfate buffer (pH 9.0) at room temperature. The reaction mixture contained 0.5 mM NAD(P)H and 43.5 μM CAA67_YL, of which about 25% contained FAD.

Results and Discussion

The present study describes the biochemical characteristics of 2-haloacrylate hydratase to understand how reduced flavin, which is essential for enzyme catalysis, is generated under physiological conditions. The enzyme from *Pseudomonas* sp. YL was heterologously expressed in *E. coli*, and a two-step purification protocol was established to obtain a homogeneous enzyme preparation sufficient for studies performed (Table 1).

As reported before[5], a homology search revealed that CAA67_YL shares 84.6% sequence identity with another 2-CAA-inducible protein from *Burkholderia* sp. WS (CAA67_WS) (accession number: BAD91550)[4]. Both proteins have an FAD-binding motif (GXGXXG) in the region from 13 to 18. The protein from *Pseudomonas* sp. YL was named 2-haloacrylate hydratase because of its ability to catalyze the hydration of 2-CAA to produce pyruvate. Although this conversion does not involve an overall oxidation or reduction, this new enzyme requires $FADH_2$ for catalysis. Further examination of the primary structure of CAA67_YL showed that the protein has an additional unusual nucleotide-binding fingerprint motif consisting of the sequence AXXGXXG in the region from 205 to 211 raising the possibility that the enzyme binds to NADH or NADPH (Fig. 1). It was reported that the nucleotide-binding domain

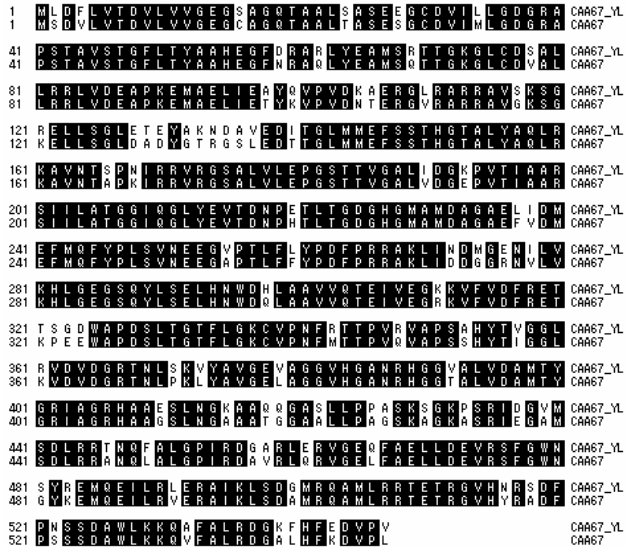


Fig. 1 Amino acid sequence alignment of CAA67_YL and CAA67_WS. Amino acid sequences of CAA67_YL and CAA67_WS were aligned by the ClustalW method using DNASTAR software (DNASTAR Inc., Madison, WI). The black boxes show the conserved residues. The numbers on the left side are the residue numbers of each amino acid sequence.

Table 1 Purification of CAA67_YL from recombinant *E. coli* cells. The enzyme activities were determined by measuring halide ions released from 2-CAA.

Purification step	Total activity	Total protein	Specific activity	Yield	Purification
	units	mg	units/mg	%	fold
Crude extract	69	140	0.49	100	1
DEAE-Toyopearl	41	34	1.2	59	2.4
Butyl-Toyopearl	30	19	1.6	43	3.3

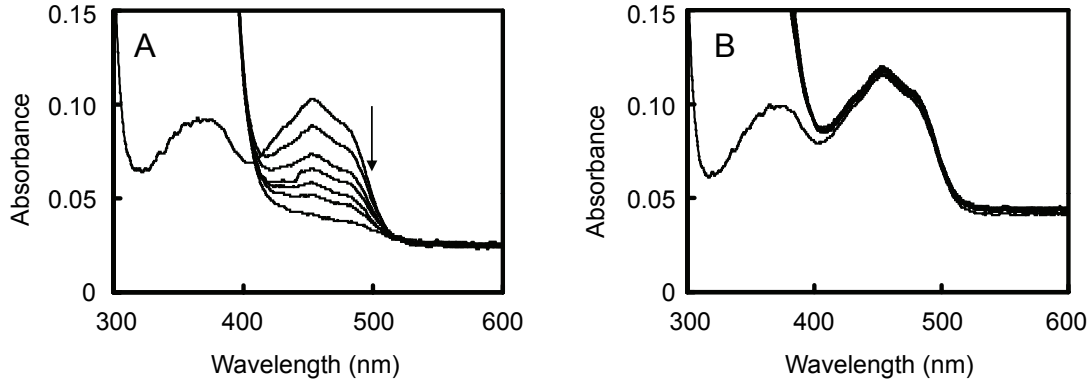
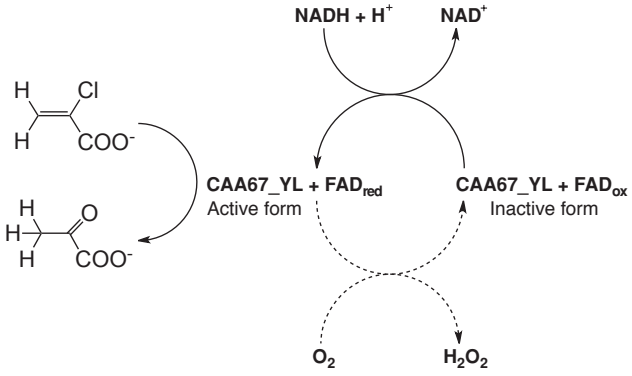


Fig. 2 Changes in the UV-visible spectrum of CAA67_YL-bound FAD in a reaction mixture containing 0.5 mM NAD(P)H. Incubation was carried out as described in the Materials and Methods in a Tris sulfate buffer pH 9.0. (A) CAA67_YL (43.5 μM) was added to the reaction mixture containing 0.5 mM NADH. (B) CAA67_YL (43.5 μM) was added to the reaction mixture containing 0.5 mM NADPH. About 25% of CAA67_YL used in this experiment contained FAD. The changes in the spectrum were recorded at 3-min intervals (top to bottom as indicated by the arrow).

along with the consensus amino acid sequence AXXGXXG or GXXGXXG preferentially binds to the NADPH cofactor[8, 9]. This motif has been found in 2-haloacrylate reductase[4] and quinone oxidoreductase[8], both of which catalyze NADPH-dependent redox reactions. Because CAA67_YL possesses AXXGXXG, it was predicted to be specific to the cofactor NADPH. However, oxidized FAD in its bound form was readily reduced by CAA67_YL at the expense of NADH (Fig. 2). The spectrum of the oxidized form of FAD is characterized by two peaks at 360 nm and 450 nm. The reduction of FAD in the presence of NADH is evident from the decrease in the absorbance at 450 nm as shown in Fig. 2A. (The absorbance around 360 nm was increased by the addition of NADH, which has a strong absorbance in this region.) The reductase activity was completely abolished when NADPH was used instead of NADH (Fig. 2B). In contrast to FAD, reduction of FMN was not catalyzed by CAA67_YL: The absorbance at 450 nm, which is characteristic of oxidized FMN, did not change by the addition of NAD(P)H (data not shown). It is reasonable that CAA67_YL catalyzes the reduction of FAD, but not the reduction of FMN, considering that $FADH_2$, but not $FMNH_2$, serves as a cofactor for the 2-haloacrylate hydratase activity of CAA67_YL[5].

Fungal chorismate synthases are bifunctional enzymes because of their intrinsic FMN: NADPH oxidoreductase activity. Other chorismate synthases from eubacteria and higher plants rely on an external source to produce reduced FMN[10, 11]. It was recently shown that there are two distinct types of type 2 isopentenyl diphosphate:dimethylallyl diphosphate isomerases. One of these was shown to catalyze NAD(P)H-dependent FMN reduction[12, 13].

From the results represented in this study, the metabolism of 2-CAA in *Pseudomonas* sp. YL can be summarized as in Scheme 1. The presence of 2-CAA in the culture medium leads to the inducible production of 2-haloacrylate hydratase, which binds to FAD. At the expense of NADH, the enzyme catalyzes the reduction of FAD and leads to the generation of the active form of the enzyme. The enzyme containing $FADH_2$ catalyzes the hydration of 2-CAA to produce 2-chloro-2-hydroxypropionic acid, which is chemically unstable and spontaneously dechlorinated to produce pyruvate and HCl. Because the redox state of the flavin cofactor is crucial for 2-haloacrylate hydratase activity, it can be envisaged that the redox balance in the cell may play a critical role in regulating the generation of the active enzyme.



Scheme 1 A proposed pathway for the metabolism of 2-chloroacrylate in *Pseudomonas* sp. YL.

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