Cloning and expression of the gene for periplasmic poly(vinyl alcohol) dehydrogenase from *Sphingomonas* sp. strain 113P3, a novel-type quinohaemoprotein alcohol dehydrogenase

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A gene for periplasmic poly(vinyl alcohol) (PVA) dehydrogenase (PVADH) was cloned, based on the N-terminal amino acid sequence of the purified PVADH from *Sphingomonas* sp. 113P3 and the sequence of the gene for PVADH (*pvaA*, GenBank accession no. AB190288). The recombinant PVADH tagged with hexahistidine was expressed in *Escherichia coli* and purified to homogeneity. The recombinant enzyme had the same characteristics as the purified enzyme from *Sphingomonas* sp. strain 113P. In addition to PVA, the recombinant PVADH could oxidize glycols such as polypropylene glycols and 1,3-butane/cyclohexanediol and 2,4-pentanediol, but neither primary nor secondary alcohols. The amino acid sequence of the recombinant PVADH showed similarity with those of PVADH from *Pseudomonas* sp. strain VM15C, putative PVADHs from *Azoarcus* sp. EbN1, and *Xanthomonas* species (54–25 % identity), and the quinohaemoprotein alcohol dehydrogenases (QH-ADHs) from *Comamonas testosteroni*, *Ralstonia eutropha* and *Pseudomonas putida* (25–29 % identity). PVADHs from strains 113P3 and VM15C have a conserved superbarrel domain (SD), probable PQQ-binding amino acids in the SD and a haem-binding domain (HBD) (they should be designated QH-PVADHs), but the positions of the amino acid sequences for the HBD and SD are the reverse of those of QH-ADHs. A protein structure of QH-PVADHs is proposed. Results of dot-blot hybridization and RT-PCR indicated that the three genes encoding oxidized PVA hydrolase, PVADH and cytochrome *c* are expressed constitutively and form an operon.

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INTRODUCTION

The water-soluble synthetic polymer poly(vinyl alcohol) (PVA), used in paper and textile mills and in copolymers as a biodegradable segment, is the only known xenobiotic carbon-chain polymer to biodegrade at high molecular weights (Kawai, 1995). As described in our previous paper (Klomklang *et al.*, 2005), its microbial degradation has been studied by several groups. Although the role of PVA oxidase could not be ruled out, PVA dehydrogenase (PVADH) and

oxidized PVA hydrolase (OPH) (both membrane-associated proteins) were thought to be the major metabolic enzymes in the degradation of PVA by *Pseudomonas* sp. VM15C (Shimao *et al.*, 1996, 2000). The partially purified PVADH from the membrane had high dehydrogenase activity toward secondary alcohols and 2,3-butanediol, 2,5-hexanediol and 1,2,6-hexanetriol (Shimao *et al.*, 1986). Another PVA-utilizing pseudomonad, *Pseudomonas* sp. 113P3, was isolated by Hatanaka *et al.* (1995a, b, 1996) and its PVADH was purified from cell-free extract and characterized as a quinohaemoprotein. The enzyme had activity on PVA, and glycols such as 1,3-butanediol, 1,3-cyclohexanediol and 2,4-pentanediol, but not on primary and secondary alcohols. Klomklang *et al.* (2005) reidentified the strain as *Sphingomonas* sp. 113P3, purified OPH and cloned the gene for it (*oph*). They revealed that PVADH and OPH were periplasmic enzymes, which were expressed constitutively. Two genes involved in PVA degradation, *oph* and *pvaA*

Abbreviations: OPH, oxidized PVA hydrolase; PPG, polypropylene glycol; PQQ, pyrroloquinoline quinone; PVA, poly(vinyl alcohol); PVADH, poly(vinyl alcohol) dehydrogenase; QH-ADH, quinohaemoprotein alcohol dehydrogenase; QH-PVADH, quinohaemoprotein poly(vinyl alcohol) dehydrogenase.

Sequence alignments of type II QH-PVADHs with type II QH-ADHs are available as supplementary data with the online version of this paper.

(encoding PVADH) were located in tandem with only a short intergenic region, and a gene for cytochrome *c* was located downstream of *pvaA*. On the other hand, Matsumura *et al.* (1998, 1999) purified and characterized a membrane-associated pyrroloquinoline quinone (PQQ)-dependent PVADH from PVA-utilizing *Alcaligenes faecalis* KK314. The purified PVADH had the same molecular mass as those from *Sphingomonas* sp. 113P3 and *Pseudomonas* sp. VM15C and included haem (S. Matsumura, Keio University, personal communication). Thus PVADHs from various PVA-utilizing bacteria have been shown to be quinohaemo-proteins, located in the periplasm or membrane-associated.

In this study, we cloned *pvaA* from *Sphingomonas* sp. strain 113P3, and the enzyme was expressed in *Escherichia coli* and purified for characterization. The homology of the recombinant enzyme with other PVADHs and quinohaemo-protein alcohol dehydrogenases (QH-ADHs) and its structure as a novel-type QH-ADH are discussed together with the presence of the *pva* operon.

METHODS

Materials. PVA117 [number-average molecular mass 75 000] used in this study was a product of the Kuraray Co. Polypropylene glycols (PPGs; diol type, 400 and 700) and PQQ were purchased from Wako Pure Chemical Industries. HisTrap HP, HiTrap Desalting, HiTrap Q HP and Sephacryl S-100 columns were purchased from Amersham Pharmacia. T4 DNA ligase, Ex Taq DNA polymerase and restriction endonucleases were products of Takara Bio Co. All other chemicals were commercial products of the highest grade available.

Bacterial strains and cultivation. *Sphingomonas* sp. strain 113P3 was used throughout this study. The strain has been deposited in the International Patent Organism Depository (IPOD) (Tsukuba, Japan) under the accession number FERM P-13483. The strain was grown on PVA medium (pH 7.5) as reported previously (Hatanaka *et al.*, 1995a). Glucose medium contained the same components as PVA medium except that glucose was added instead of PVA117. The cells were harvested by centrifugation at 8000 *g* for 30 min, washed twice with 0.85% NaCl and kept at -80°C until use. *E. coli* DH5 α as the cloning host and *E. coli* transformants were grown at 37°C in LB medium, supplemented with 50 μg ampicillin ml^{-1} when necessary.

Cloning and expression of *pvaA*. DNA purification, transformation and electrophoresis were performed as described by Sambrook & Russell (2001). Ex Taq DNA polymerase was routinely used for PCR under the conditions recommended by the manufacturer. The PCR products were sequenced for both strands. The primers were designed based on the N-terminal amino acid sequence (APQSGHAVPADQLD, unpublished data) of the purified enzyme (Hatanaka *et al.*, 1995b) and the sequence of *pvaA* (Klomklang *et al.*, 2005; GenBank accession number AB190288). The following primers were used: forward, 5'-GGAATTCATATGGGCTCCCATGCTTGG-3'; reverse, 5'-ATTTGCGGCCGCTTTTCCATCGACCGAGAAGGC-3' (*NdeI* and *NotI* restriction enzyme sites underlined). The amplified fragment (PCR product) was purified and ligated into pCR2.1 plasmid vector using the TA cloning kit (Invitrogen). The plasmid was transformed into the host and extracted from transformant *E. coli*, which was sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems) and a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instruction

manuals. The fragment was digested with *NdeI* and *NotI* and then ligated into the corresponding position of a pET-23a vector. The resultant plasmid (pET-pvaA) was transformed into *E. coli* strain BL21(DE3)pLysS, an expression host (Novagen). The transformants were grown in LB medium containing 50 μg ampicillin ml^{-1} and 1% glucose at 37°C and cultured to an OD₆₀₀ of 0.6. IPTG and δ -aminolaevulinic acid (a precursor for haem) were added at 10 μM and 1 mM, respectively (Sugiura *et al.*, 1996). Then the temperature was shifted to 20°C and shaking was performed at 100 r.p.m. for 3 h. Bacterial pellets obtained from a 1 litre culture were suspended in an appropriate amount of 50 mM Tris/HCl buffer (pH 8.0) containing 0.5 M NaCl and sonicated with a high-intensity BC-130PB ultrasonic processor (Sonics & Materials) at 10 s intervals for a total time of 10 min at 20 kHz. The sonicated cell suspension was centrifuged at 12 000 *g* for 30 min to remove the cell debris and unbroken cells. The resultant supernatant was used as cell-free extract.

Assay of PVADH. The reaction mixture contained 1 mM PVA117 (7.5 mg), 1 mM phenazine ethosulfate, 0.2 mM 2,6-dichlorophenol-indophenol (DCIP), 1 mM KCN, 1 mM CaCl_2 , 3 μM PQQ, 50 mM potassium phosphate buffer (pH 7.2), and an appropriate amount of the enzyme in a total volume of 1 ml. The reaction was started by the addition of the enzyme solution preincubated with more than an equivalent molar concentration of PQQ (based on the monomer protein) and CaCl_2 at 37°C for 10 min. The enzyme reaction was done at 37°C and the initial reaction rate was measured by the decrease in A_{600} due to reduction of DCIP ($\epsilon_{600} = 1.91 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) with a Shimadzu UV-160 spectrophotometer with a 1 cm light path. A reaction mixture without substrate was used as a reference. One unit of enzyme was defined as the amount of enzyme that reduced 1 μmol DCIP min^{-1} under the assay conditions. The specific activity of the enzyme was expressed as units (mg protein) $^{-1}$.

Purification of the recombinant PVADH. All buffers used for purification were kept at 4°C . The cell-free extracts were further subjected to filtration with a 0.45 μm pore size filter and the resultant supernatant was applied on a HisTrap HP column (3 $\times$ 1 ml, connected in series) (Amersham Biosciences) under a LC-9A liquid chromatography system (Shimadzu). PVADH was eluted with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 100 mM imidazole at a flow rate of 1 ml min^{-1} . The active fractions were pooled and desalted through a HiTrap Desalting column (3 $\times$ 5 ml, connected in series) (Amersham Biosciences) using 10 mM Tris/HCl buffer (pH 8.0). The desalted sample was put on a HiTrap Q HP column (2 $\times$ 1 ml, connected in series) (Amersham Biosciences) equilibrated with the same buffer as used for desalting. Elution was done stepwise with 20 mM Tris/HCl buffer (pH 8.0) containing 50–1000 mM NaCl. PVADH was eluted with 20 mM Tris/HCl buffer (pH 8.0) containing 500 mM NaCl. The active fractions were collected and desalted with a HiTrap Desalting column (5 ml) (Amersham Biosciences). The resultant enzyme solution was used as the purified enzyme, which was stable for more than a month at 4°C . As the purified enzyme did not show any activity toward PVA in the absence of PQQ, the expressed protein was thought to be an apo-PVADH.

Analyses. The protein concentration was determined by a CBB protein assay reagent kit (Nacalai Tesque) with bovine serum albumin as the standard. The homogeneity of the protein and the molecular mass of the enzyme subunit were confirmed by SDS-PAGE based on the method of Laemmli (1970). The molecular mass of the native enzyme was determined by gel filtration on a Sephacryl S-100 column (1 $\times$ 90 cm) using a gel filtration calibration kit (Amersham Biosciences). Absorption spectra were measured with a BioSpec-1600 spectrophotometer (Shimadzu). The N-terminal amino acid sequence of the purified enzyme was determined with a Procise 491 protein sequencer (Applied Biosystems). Homology searches were performed with the BLAST program (<http://blast.genome.jp/>).

Table 1. Primers

Primer	Sequence (5'→3')	Description
PVADH-NdeI	GGAATTCCATATGGGCTCCCATGCTTGG	See Fig. 4
PVADH-NotI	ATTTGCGGCCGCTTTTCCATCGACCGAGAAGGC	See Fig. 4
OPH-F	CAAGTACAAGACCGATGCGG	5' of <i>oph</i>
OPH-R	ACAGTGATCACGACCATCGG	3' of <i>oph</i>
PVADH-F	GATGACGGCAAGTCTATGC	5' of <i>pvaA</i>
PVADH-R	TGGACATAGACCATTCCGCC	3' of <i>pvaA</i>
CytC-F	GATGTGCAGCTTGCCATTCCG	5' of <i>cytC</i>
CytC-R	CGTCAGACAGGCAATGATCG	3' of <i>cytC</i>

Accession numbers used for alignment. Sequences of PVADHs from databases were used for alignment of amino acid sequences: AB190288, *Sphingomonas* sp. strain 113P3; AB008494, *Pseudomonas* sp. strain VM15C; CR555308, *Azoarcus* sp. EbN1; AE012071, *Xanthomonas axonopodis*; AE012531, *Xanthomonas campestris*; and AE013598, *Xanthomonas oryzae*. Sequences for ADHs used for alignment of QH-ADHs and PVADHs were from X81880 (*Comamonas testosteroni*), AF277373 (*Ralstonia eutropha*, tetrahydrofurfuryl ADH), AB091400 (*Pseudomonas putida*, ADH IIB) and AB204833 (*P. putida*, ADH IIG). The program used for alignment was GENETYX-WIN version 5.0.

Preparation of total RNA and reverse transcriptase-PCR (RT-PCR). *Sphingomonas* sp. strain 113P3 was grown at 30 °C on PVA or glucose medium to mid-exponential phase. Total RNA was isolated with ISOGEN (Nippon Gene Co.). The RNA samples were further treated with DNase I (Invitrogen). RT-PCR was performed with a One-Step RT-PCR kit (Qiagen) according to the supplier's instructions. Negative control experiments were done by omitting the reverse transcription step. The sequences of primers used for each PCR amplification are listed in Table 1.

RNA dot-blot hybridization. Three micrograms of total RNA of each sample was spotted and heat-fixed on a nylon membrane (Hybond-N+, Amersham Biosciences) at 80 °C for 30 min. Probes were amplified by PCR with appropriate primers listed in Table 1 and labelled as described in the digoxigenin system manual (Boehringer Mannheim Biochemicals). The conditions used for pre-hybridization, hybridization, washing and detection followed the manufacturer's recommendations.

RESULTS

Cloning and expression of *pvaA*

A fragment of 1962 bp was amplified from genomic DNA by PCR with the designed primers. The fragment was cloned into a pCR2.1 vector and the plasmid sequence was

confirmed to be the same as that of *pvaA* under accession no. AB190288. *E. coli* BL21(DE3)pLysS expressed the recombinant enzyme tagged with hexahistidine and the enzyme was recovered from cell-free extracts. The protein was purified about 44-fold, to show a specific activity of 18.0 (Table 2). The N-terminal amino acid sequence of the recombinant enzyme was in accordance with that of the purified enzyme from *Sphingomonas* sp. 113P3.

Characterization of the purified recombinant PVADH

The purity and the molecular mass of the purified enzyme were confirmed on SDS-PAGE to give a single band of approximately 68 kDa (Fig. 1). The molecular mass of the native enzyme on gel filtration was estimated to be approximately 68 kDa. This suggested that the enzyme is a monomeric protein. The optimal pH and temperature of the recombinant PVADH were 7.2 (potassium phosphate buffer) or 7.4 (Tris/HCl buffer) and around 37 °C, respectively. The enzyme displayed activity only when preincubated with PQQ. One mole of PQQ was bound to one mole of apoenzyme, which was confirmed by the method described by Hatanaka *et al.* (1995a). The purified enzyme showed distinct absorption peaks at 420, 520 and 550 nm in the presence of PQQ, similar to those of PQQ-dependent alcohol dehydrogenase (ADH) from *Comamonas testosteroni* ATCC 15667 (Stoorvogel *et al.*, 1996) (Fig. 2). Peaks indicated the presence of haem *c* and an electron flow from PQQ to haem *c*. The enzyme had higher activity toward PPGs (137 % for PPG 400 and 172 % for PPG 700) than PVA 117 (100 %), which corresponded to the activity of PPG dehydrogenase on PPG (diol type) 700 (100 %) and PVA 500 (41 %) (Tachibana *et al.*, 2003). The enzyme also acted

Table 2. Purification of the recombinant PVADH

Purification step	Protein (mg)	Total activity (units)	Specific activity (units mg ⁻¹)	Purification (fold)	Yield (%)
Cell-free extracts	239	98	0.41	1	100
HisTrap HP	5.5	66	12.0	29	67
HiTrap Desalting	3.8	61	16.0	39	62
HiTrap Q HP	3.0	54	18.0	44	55

Fig. 1. SDS-PAGE (with 10% acrylamide gel) of the recombinant PVADH. A, cell-free extracts, prepared from cells incubated with IPTG (+) and without IPTG (-); B, active fractions obtained by HisTrap HP column chromatography; C, active fractions obtained by HiTrap Q HP column chromatography; M, molecular markers. The arrow indicates PVADH.

slightly on glycols such as 2,4-pentanediol (1.6%), 1,3-cyclohexanediol (8.4%) and 1,3-butanediol (1.0%), but not at all on secondary alcohols (2-pentanol, 2-hexanol and 4-heptanol) and primary alcohols (methanol to 1-pentanol). Thus, PVADH can recognize $\text{-O-CH}_2\text{CH}(\text{CH}_3)\text{OH}$ and $\text{-CH}(\text{OH})\text{CH}_2\text{CH}(\text{OH})\text{-}$. Higher activity toward PPGs is probably due to the closer distance of ether oxygen and a secondary alcohol group in PPGs than that of two secondary alcohol groups in PVA. Ca^{2+} and Mg^{2+} enhanced enzyme activity markedly, but the activation by Mg^{2+} was less than 80% of the activation by Ca^{2+} , a finding similar to that for ADH from *C. testosteroni* ATCC 15667 (Groen *et al.*, 1986). All these characteristics were in accordance with those of the purified enzyme from *Sphingomonas* sp. 113P3 (Hatanaka *et al.*, 1995b) except that activity toward PPG was newly found.

Amino acid sequences of PVADHs

Database searching indicated several homologous proteins from other origins, as shown in Fig. 3. Apart from the PVADHs from *Sphingomonas* sp. strain 113P3 and *Pseudomonas* sp. strain VM15C, the function of proteins encoded by the homologous genes from *Azoarcus* sp. EbN1 and *Xanthomonas* species are unknown. They showed 25–54% identity with the amino acid sequence of *pvaA* from *Sphingomonas* sp. strain 113P3. PVADH of strain 113P3 had the highest identity (54%) to that of *Pseudomonas* sp. strain VM15C, but others had low identities of 25–33%. PVADHs from *Sphingomonas* sp. strain 113P3, *Pseudomonas* sp. strain

Fig. 2. Absorption spectra of the purified recombinant enzyme. The absorption spectrum was measured with the purified enzyme (150 µg) dissolved in 10 mM potassium phosphate buffer (pH 7.2) containing 1 mM CaCl_2 , which was incubated at 37 °C for 10 min with and without more than a stoichiometric amount of PQQ, and then PVA (7.5 mg ml⁻¹; 1 mM) was added. Water was used as blank.

VM15C and *Azoarcus* sp. EbN1 conserved a haem-binding motif (CxxCH), and the domain including this motif has similarity to the haem-binding domain of QH-ADHs. A probable PQQ-binding amino acids motif and a superbarrel domain made up of eight beta sheets (called W1–W8), which are common to type II QH-ADHs (Toyama *et al.*, 2004), were also found in three PVADHs. On the other hand, those from *Xanthomonas* species conserved a probable PQQ-binding amino acids and a superbarrel domain, but lacked a haem-binding motif: the presence of a probable PQQ-binding amino acids motif and a superbarrel domain is characteristic of type I quinoprotein (Q)-ADHs. These results suggested that PVADHs are divided into two classes, type I Q-ADHs and type II QH-ADHs, which should be specifically called type I Q-PVADH and type II QH-PVADH, respectively.

Dot-blot hybridization and RT-PCR

Our previous work (Klomklang *et al.*, 2005) indicated that PVADH and oxidized PVA hydrolase were constitutively expressed. To confirm transcription of the *pvaA* gene, strain 113P3 was cultivated on PVA or glucose medium. Total RNAs were isolated and treated with DNase I until no band could be detected by routine PCR. Dot-blot hybridization was analysed with three DNA fragments including the *oph*, *pvaA* and *cytC* genes involved in the gene cluster. The results clearly showed that each gene was expressed constitutively in

Fig. 3. Sequence alignment of PVADHs from different sources. Identities with PVADH from *Sphingomonas* sp. strain 113P3 are 54% (*Pseudomonas* sp. strain VM15C), 33% (*Azoarcus* sp. EbN1) and 25% (*Xanthomonas* species). Bold type is used to indicate the tryptophan-docking motifs (AxD/NxxTGKxW) made up of the outer C and D strands of the eight beta sheets (W-motifs) that compose the superbarrel structure. Probable PQQ-binding amino acids are shown with a dark grey background, and those involved in haem c-binding with a light grey background.

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Fig. 4. Dot-blot hybridization and RT-PCR analysis of the *pva* operon. (a) Dot-blot hybridization was performed using gene-specific probes generated by PCR. The sizes of the probes were identical to products (1), (3) and (5) obtained by RT-PCR analysis, as shown in (b). (b) RT-PCR analysis of each gene and intergenic region. The sizes of the detected PCR bands are illustrated by double arrows (1)–(5) above the illustration of gene structure. M, 1 kb ladder marker; –, negative control prepared by omitting the reverse-transcription step using an RNA sample prepared from either PVA-grown cells or glucose-grown cells; +, RNAs were isolated from either PVA-grown cells or glucose-grown cells; PVA, RNAs from PVA-grown cells; Glu, RNAs from glucose-grown cells.

both media (Fig. 4). Transcription of each gene was confirmed by RT-PCR with gene-specific primers. Constitutively expressed RNA gave the same specific products as those amplified using a genomic DNA as a template. Interestingly, two intergenic regions among the three genes (*oph*, *pvaA* and *cytC*) could be amplified. These results suggested that the three genes in the same orientation were transcribed as a single operon (designated the *pva* operon) and constitutively expressed.

DISCUSSION

Quinoprotein ADHs are subdivided into three groups (Toyama *et al.*, 2004). Type I are simple quinoproteins having no haem (Q-ADHs), while type II (soluble periplasmic) and type III (membrane-bound) are quinohaemoproteins (QH-ADHs). Type II QH-ADH is soluble in the periplasm and has a relatively wide distribution among several Proteobacteria. Type III QH-ADH is membrane-bound, working on the periplasmic surface,

and is unique to acetic acid bacteria. PVADHs have been purified and characterized from three PVA-utilizing strains: *Pseudomonas* sp. strain VM15C (membrane-associated; Shimao *et al.*, 1986, 1996), *Alcaligenes faecalis* KK314 (membrane-associated; Matsumura *et al.*, 1998, 1999) and *Sphingomonas* sp. strain 113P3 (Hatanaka *et al.*, 1995a) (periplasmic; Klomklang *et al.*, 2005). All of them were found to be QH-ADHs categorized as type II (Toyama *et al.*, 2004). This would be reasonable, because PVADH from *Sphingomonas* sp. strain 113P3 was a periplasmic soluble enzyme having a high similarity to that from *Pseudomonas* sp. strain VM15C and PVADHs were found among three Proteobacteria. In addition, some type II QH-ADHs had activities toward PVA and the protein structure of QH-PVADH is the same as that of type II QH-ADH, but differs from that of type III QH-ADH. Database searching indicated several homologous proteins from other origins (Fig. 3): putative PVADH from *Azoarcus* sp. EbN1 conserved a haem *c*-binding domain, a likely PQQ-binding amino acids motif and a superbarrel domain made up of

eight beta-sheets (called W1–W8) similar to PVADHs from three PVA-utilizing strains, but those from *Xanthomonas* species conserved a likely PQQ-binding amino acids motif and a superbarrel domain, but lacked a haem-binding domain. On the other hand, PPG dehydrogenase from *Stenotrophomonas maltophilia* was a type I Q-ADH and oxidized PVA (41 %) as well as PPG (diol type) 700 (100 %) (Tachibana *et al.*, 2003). PVADHs from *Xanthomonas* species are thought to be type I Q-ADHs similar to PPG dehydrogenase. Thus type I Q-ADHs and type II QH-ADHs have activity toward PVA (designated type I Q-PVADHs and type II QH-PVADHs, respectively). In general, type II QH-ADHs accommodate alcohol substrates of much larger size and variety in the active site cavity than type I Q-ADHs (Toyama *et al.*, 2004). The gene for PVADH has been cloned from *Pseudomonas* sp. strain VM15C, but the product has not been purified yet (Shimao *et al.*, 1996). The *pvaA* gene encoding PVADH (Klomklang *et al.*, 2005) was cloned from *Sphingomonas* sp. strain 113P3 and expressed in *E. coli* in this study. The recombinant enzyme was purified and confirmed to be the same protein as PVADH purified from *Sphingomonas* sp. strain 113P3. The amino acid sequence of PVADH has 54 % identity to that from *Pseudomonas* sp. VM15C, but these two enzymes differ in specificity toward diols and alcohols. Activity of PVADH from strain VM15C toward PPGs has not been confirmed. The structure and function of type II QH-ADHs was well characterized by X-ray crystallography with ADH from *C. testosteroni* (Oubrie *et al.*, 2002) and ADH IIB from *P. putida* HK5

(Chen *et al.*, 2002). Type II QH-PVADHs have a structural motif common to this group, a quinoprotein-specific superbarrel domain (see the sequence alignment available as supplementary data with the online version of this paper), where PQQ is deeply embedded in the centre, and a unique haem-binding *c* domain. The position of the amino acid sequences for the superbarrel and haem-binding *c* domains in PVADHs, however, is the reverse of that in other type II QH-ADHs: the superbarrel domain exists in the N-terminal portion of QH-ADHs, but in the C-terminal portion of QH-PVADHs. Thus we can conclude that type II QH-PVADHs and QH-ADHs have a common progenitor, but their gene structures were reorganized during evolution. We do not have any evidence for their evolutionary history, but from the history and nature of the chemicals on which they act (PVA and alcohol: the former is a xenobiotic compound and the latter is a natural compound), it seems reasonable to think that type II QH-PVADHs have evolved from type II QH-ADHs.

Among type II QH-ADHs, ADH from *C. testosteroni* (Jongejan *et al.*, 1998; Duine & Kawai 1998) and tetrahydrofurfuryl ADH from *Ralstonia eutropha* (Zarnt *et al.*, 1997) were active on PEG and secondary alcohols, but ADHs IIB and IIG from *P. putida* HK5 were inactive on PEG and PVA (Toyama *et al.*, 1995), although ADHs IIB and IIG have a bigger active-site cavity volume (120 and 150 Å³, respectively) than ADH from *C. testosteroni* (62 Å³). The active site containing PQQ in the superbarrel and the

Fig. 5. Proposed protein structure for QH-PVADHs. A corresponds to a haem-binding cytochrome domain and B to a superbarrel domain. C-C indicates a Cys-Cys motif. Ns and Nh indicate N-terminal amino acids of the superbarrel domain and haem-binding cytochrome domain, respectively; Cs and Ch indicate C-terminal amino acids of the superbarrel domain and haem-binding cytochrome domain, respectively. (N) and (C) represent the N- and C-terminal amino acids of the enzyme.

haem-binding domain make the hydrophobic channel leading to the active site cavity and the haem-binding domain would move up and down a little during turnover, due to the movement of the residues in the active site during catalysis (Toyama *et al.*, 2004). The accessibility of substrates to an active site is possibly dependent not only on the size of the site, but also on the 3D structure of the hydrophobic channel. The hydrophobic channel found in ADH IIB and IIG seems to be unable to accept a whole bulky molecule of PEG or PVA. Here we propose a novel group of type II QH-ADHs conserved in PVADHs (called QH-PVADHs), where the sequences in the proteins of the haem domain and the superbarrel domain are in the reverse position compared with all the other QH-ADHs, as indicated in Fig. 5. A Cys-Cys motif relevant to electron flow from the C-5 of PQQ to the haem iron exists at the same position between the W1 and W2 motifs in QH-ADHs [Cys147-Cys148, Cys136-Cys137, Cys127-Cys128 and Cys138-Cys139 in the enzymes from *C. testosteroni*, *Ralstonia eutropha* and *Pseudomonas putida* (IIB and IIG), respectively (Toyama *et al.*, 2004)]. The motif was shifted to the same position between the W2 and W3 motifs in type II QH-PVADHs (Cys312-Cys313 and Cys307-Cys308 in the enzymes from strains 113P3 and VM15C, respectively). From the fact that QH-PVADHs conserve the same domains and motifs as type II QH-ADHs, the same protein structure as type II QH-ADHs could be predicted for type II QH-PVADHs, as shown in Fig. 5. Elucidation of difference in reactivity toward macromolecules must await X-ray crystallographic analysis of QH-PVADHs.

Dot-blot hybridization and RT-PCR showed the constitutive expression of *cytC* together with *oph* and *pvaA* as an operon, suggesting electron transfer from PQQ to haem *c* (both exist in PVADH) and then to cytochrome *c*. In pseudomonads, azurin is known as a natural electron acceptor in the periplasm. In sphingomonads cytochrome *c* might be playing the same role as azurin.

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