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Existence of a Novel Enzyme, Pyrroloquinoline Quinone-Dependent Polyvinyl Alcohol Dehydrogenase, in a Bacterial Symbiont, *Pseudomonas* sp. Strain VM15C

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A novel enzyme, pyrroloquinoline quinone (PQQ)-dependent polyvinyl alcohol (PVA) dehydrogenase, was found in and partially purified from the membrane fraction of a PVA-degrading symbiont, *Pseudomonas* sp. strain VM15C. The enzyme required PQQ for PVA dehydrogenation with phenazine methosulfate, phenazine ethosulfate, and 2,6-dichlorophenolindophenol as electron acceptors and did not show PVA oxidase activity leading to H_2O_2 formation. The enzyme was active toward low-molecular-weight secondary alcohols rather than primary alcohols. A membrane-bound PVA oxidase was also present in cells of VM15C. Although the purified oxidase showed a substrate specificity similar to that of PQQ-dependent PVA dehydrogenase and about threefold-higher PVA-dehydrogenating activity with phenazine methosulfate or phenazine ethosulfate than PVA oxidase activity with H_2O_2 formation, it was shown that the enzyme does not contain PQQ as the coenzyme, and PQQ did not affect its activity. Incubation of the membrane fraction of cells with PVA caused a reduction in the cytochrome(s) of the fraction.

Pyrroloquinoline quinone (PQQ) is a novel coenzyme which was discovered in methanol dehydrogenases (8), and its structure was determined in 1979 (24). So far, several types of bacterial dehydrogenases (2, 3, 5, 6–9, 12–16, 19) and even fungal and mammalian amine oxidases (4, 20) have been found to be quinoproteins which contain PQQ as the coenzyme. Thus, it is becoming clear that PQQ is an important coenzyme involved in various oxidative metabolic processes. In our previous studies (23, 25–31) on polyvinyl alcohol (PVA)-utilizing mixed cultures of two bacterial symbionts, *Pseudomonas putida* VM15A and *Pseudomonas* sp. strain VM15C, it was found that symbiotic PVA utilization is based on cross-feeding of PQQ from VM15A to the PVA-degrading bacterium VM15C. This has shown PQQ as a vitamin for the first time. PQQ was effective not only in causing growth of VM15C on PVA but also in enhancing the growth rate and cell yield. All PVA-degrading *Pseudomonas* sp. strains isolated by us also required PQQ as an essential growth factor for PVA utilization, as in the case of VM15C. On the other hand, PQQ was not required by VM15C when the strain was grown on carbon sources other than PVA. These facts indicate the possibility that a PQQ-dependent enzyme catalyzes a specific and significant reaction in bacterial PVA metabolism.

To investigate PQQ participation in bacterial PVA metabolism, a search for a PQQ-dependent enzyme was carried out in VM15C cells, and a novel enzyme, PQQ-dependent PVA dehydrogenase, was found in its membrane in this work. This report concerns this finding and some properties of the enzyme. PVA-oxidizing enzymes found so far in PVA-degrading bacteria have been only PVA oxidases (22, 30, 32). As to the membrane-bound PVA oxidase of VM15C, it was clarified in this work that the enzyme does not contain PQQ as the coenzyme. Furthermore, we obtained evidence suggesting that PVA oxidation on the cytoplasmic membrane is coupled with the electron transport chain.

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MATERIALS AND METHODS

Materials. Authentic PQQ and [^{14}C]PQQ were kindly supplied by M. Ameyama, Yamaguchi University; they were purified from the culture supernatant of a methylotrophic bacterium grown on methanol and [^{14}C]methanol, respectively (4). The specific radioactivity of [^{14}C]PQQ was 0.554 Ci/mol. PVA was purchased from Wako Pure Chemical Industries, Osaka, Japan, and washed with methanol in a Soxhlet extractor. PVA with a degree of polymerization of 1,500 was used for enzyme assays and that with a degree of polymerization of 500 was used for cultivation.

Microorganisms and growth conditions. *Pseudomonas* sp. strain VM15C was maintained on nutrient agar slants and used throughout this work. The basal medium was the same as that previously described (23). PVA, peptone (Difco Laboratories, Detroit, Mich.), and glucose were used as sole carbon sources at 5 g/liter.

The *Acinetobacter calcoaceticus* mutant lacking glucose dehydrogenase activity used was the same as that previously described (31); it produces apo-glucose dehydrogenase but not the coenzyme, PQQ. The medium was composed of 1% peptone (Difco Laboratories), pH 7.3.

Cultivation was carried out with 1 liter of medium in 2-liter flasks at 30°C with reciprocal shaking.

Enzyme assays. Unless otherwise noted, the assay mixture for PVA oxidase activity contained 150 μmol of potassium phosphate buffer (KPB; pH 7.5), 0.25 μmol of 4-aminoantipyrine, 3.2 μmol of phenol, 18 U of peroxidase (type I; Sigma Chemical Co., St. Louis, Mo.), 22.5 mg of PVA, and the enzyme preparation in a total volume of 3 ml. The reaction was started by the addition of PVA. The rate of formation of H_2O_2 was determined at 30°C from the increase in A_{500} resulting from oxidative coupling of H_2O_2, 4-aminoantipyrine, and phenol (1), with 6.39 as the millimolar absorption coefficient corresponding to H_2O_2. One unit of activity was defined as the amount which produced 1.0 μmol of H_2O_2 per min under the assay conditions.

Unless otherwise noted, the assay mixture for PVA dehy-

drogenase activity contained 150 μmol of KPB (pH 7.5), 3 μmol of phenazine ethosulfate (PES), 0.3 μmol of 2,6-dichlorophenolindophenol (DCPIP), 3 μmol of KCN, 22.5 mg of PVA, and the enzyme preparation in a total volume of 3 ml. The reaction was started by the addition of PVA. The rate of reduction of PES was determined at 30°C from the decrease in A_{600} resulting from coupled reduction of DCPIP, with 19.1 as the millimolar absorption coefficient of DCPIP (10). One unit of activity was defined as the amount which reduced 1 μmol of DCPIP per min under the assay conditions. When phenazine methosulfate (PMS) was used instead of PES, or only DCPIP was used as an electron acceptor, the assay was performed in the same way. When NAD, NADP, and coenzyme Q's (Sigma Chemical Co.) were used instead of PES and DCPIP, reduction of NAD and NADP was followed at 340 nm, and that of coenzyme Q's was followed at 275 nm (21). When flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were used, their reduction was determined at 450 nm under an N_2 atmosphere.

When the enzyme activity was assayed after preincubation with and without PQQ, each sample was preincubated at 30°C for a given time in 50 mM KPB (pH 7.5) in a total volume of 2.2 ml, followed by addition of reagents for the assays of enzyme activities to a final volume of 3 ml.

Preparation of the cell-free extract, membrane fraction, and solubilized membrane of VM15C. Cells were harvested and washed twice with 50 mM KPB (pH 7.5) by centrifugation at 4°C and then stored at −20°C. The frozen cell cake was suspended in the same buffer to 10% (wt/vol), and disrupted with an ultrasonic oscillator (19 kHz) for 15 min at 0 to 5°C. The cell debris was removed by centrifugation at 15,000 × *g* for 30 min at 4°C. The supernatant was used as the cell-free extract. The cell-free extract was further centrifuged at 100,000 × *g* for 60 min at 4°C. The sediment was used as the membrane fraction of cells. The membrane fraction was suspended in 50 mM KPB (pH 7.5) containing Triton X-100 and gently stirred at 5°C for 16 h. The suspension was centrifuged at 100,000 × g for 60 min at 4°C. The supernatant was used as the solubilized membrane. The membrane fraction was not washed before the solubilization, since it was confirmed in a small-scale experiment that the enzyme activities of the fraction changed negligibly by washing with 50 mM KPB (pH 7.5).

Purification of membrane-bound PVA oxidase. Membrane-bound PVA oxidase was purified by a method similar to that described previously (30) from the solubilized membrane, which was prepared with 1% Triton X-100 from VM15C cells grown in PVA medium supplemented with PQQ at 5 μg/liter for 4 days.

Purification of PQQ-dependent PVA dehydrogenase. All operations were performed at 5°C. Solubilized membrane prepared with 2% Triton X-100 from VM15C cells (wet weight, 20 g) grown in PVA medium supplemented with PQQ at 10 μg/liter for 4 days was dialyzed against 10 mM Tris hydrochloride (pH 8.5) containing 0.1% Triton X-100 and then applied to a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column (2.5 by 20 cm) equilibrated with the same buffer. After the column was washed with equilibration buffer, PQQ-dependent PVA dehydrogenase was eluted with a linear concentration gradient of KCl in the buffer. The active fractions were collected and dialyzed against 10 mM KPB (pH 7.0) containing 0.1% Triton X-100 and used as the partially purified enzyme.

PQQ assay. The membrane fraction of the *A. calcoaceticus* mutant was prepared, by a method similar to that used for VM15C, from cells grown in the medium for 24 h and used as the apo-glucose dehydrogenase preparation for the PQQ assay. The sample and the apoenzyme (1 mg as protein) were incubated at 25°C in 33.3 mM KPB (pH 6.0) containing 0.5 μmol of $MgCl_2$ for 30 min in a total volume of 2.6 ml, followed by addition of 0.3 μmol of PMS, 0.5 μmol of DCPIP, 3 μmol of KCN, and 100 mmol of glucose to a final volume of 3 ml. The rate of reduction of DCPIP was measured at 600 nm at 25°C. PQQ contents of samples were determined from the linear relationship between the DCPIP reduction rate and authentic PQQ (0.5 to 5 ng) in the assay mixture.

Measurement of radioactivity. Radioactivity was measured with a liquid scintillation system (LSC-703; Aloka Co., Tokyo, Japan) and a scintillant which contained 6 g of 2,5-diphenyloxazole and 0.5 g of *p*-bis(*o*-methylstyryl)benzene per liter of a mixture of toluene and Triton X-100 (66.7/33.3%, vol/vol).

Isoelectric focusing. Isoelectric focusing was performed according to the method of Vesterberg (34) with a 110-ml column (LKB, Bromma, Sweden). The electrophoresis medium contained Ampholine (LKB) with a pH range of 3.5 to 10 and 0.1% Triton X-100.

Other methods. Protein was estimated with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.), using bovine serum albumin as the standard. Disc gel electrophoresis was carried out according to the method of Davis (11) in a 6.4% polyacrylamide gel with a pH 9.5 buffer system containing 1% Triton X-100. The gel was stained with Coomassie brilliant blue R-250 to detect protein. Gel filtration for estimation of the molecular weight of an enzyme was carried out on a TSK-Gel G3000SW column (0.75 by 60 cm; Toyo Soda Manufacturing Co. Ltd., Tokyo, Japan) with 0.1 M KPB (pH 7.0) containing 0.2 M NaCl and 0.1% Triton X-100 as the eluent and marker proteins (MW-marker HPLC; Oriental Yeast Co., Ltd., Tokyo, Japan) in a high-pressure liquid chromatography system (6000A pump, U6K injector, and 440 absorbance detector; Waters Associates Inc., Milford, Mass). The effluent was monitored at 254 nm.

RESULTS

Effect of PQQ on PVA-oxidizing enzyme activities of cell-free extracts. Cell-free extracts of VM15C grown in several media were preincubated with and without PQQ for 30 min, and then PVA oxidase and PVA dehydrogenase activities in the cell extracts were assayed to examine the effect of PQQ on the enzyme activities (Table 1). All of the cell extracts showed markedly higher PVA dehydrogenase activities, but slightly lower PVA oxidase activities, when they were preincubated with PQQ than without it. Preincubation without PQQ for 30 min was confirmed to negligibly affect the enzyme activities of each cell extract. Thus, it was found that PQQ enhances the PVA dehydrogenase activities of cell-free extracts of VM15C, irrespective of the growth conditions (kind of carbon sources, supplementation of PQQ to the culture media).

Membrane-bound PVA oxidase. As previously reported (30), PVA oxidase of the strain has high PVA dehydrogenase activity. To examine the effect of PQQ on the enzyme, PVA oxidase was purified from the solubilized membrane obtained from cells grown on PVA. The purified enzyme preparation showed PVA oxidase activity of 1.68 U/mg of protein and gave a single band on disc gel electrophoresis (Fig. 1). The molecular weight of the enzyme was estimated to be 31,000 by gel filtration on a TSK-Gel G3000SW column. The pI of the enzyme was found to be 5.4 on

TABLE 1. Effect of PQQ on PVA oxidase and PVA dehydrogenase activities of cell-free extracts of *Pseudomonas* sp. strain VM15C

Growth conditions[a]	Activity (mU/mg of protein)			
	PVA oxidase		PVA dehydrogenase	
	−PQQ[b]	+PQQ[c]	−PQQ	+PQQ
PVA + PQQ	3.2	2.2	13.1	33.8
Peptone + PQQ	12.8	10.3	49.1	102.9
Peptone	14.8	11.9	48.7	104.4
Glucose + PQQ	2.2	1.7	14.7	37.8
Glucose	3.3	2.5	9.2	26.2

[a] VM15C was cultivated in PVA, peptone, and glucose media suplemented with PQQ at 10 μg/liter and in peptone and glucose media without PQQ for 4 days.

[b] After preincubation without PQQ for 30 min, the activity was assayed.

[c] After preincubation with 1.36 μM PQQ for 30 min, the activity was assayed.

isoelectric focusing. The enzyme showed high oxidase activity toward some low-molecular-weight secondary alcohols but not primary alcohols (Table 2). These properties of the purified membrane-bound PVA oxidase were essentially the same as those of PVA oxidases purified as extracellular enzymes from the culture supernatants of other PVA-degrading bacteria (22, 32).

Effect of PQQ on membrane-bound PVA oxidase. PVA-dehydrogenating activity of the purified membrane-bound PVA oxidase was examined with several electron acceptors, after incubation with and without PQQ (Table 3). In either case, i.e., with or without PQQ, the enzyme utilized PES, PMS, and DCPIP besides O_2 for PVA dehydrogenation, and the activities with PES and PMS were about threefold higher than the PVA oxidase activity leading to H_2O_2 formation. However, it was noted that PQQ did not affect PVA dehydrogenation by the purified enzyme with any electron acceptor.

PVA dehydrogenase activity of the solubilized membrane was enhanced by PQQ similarly to that of cell-free extracts. Due to the fact that PQQ stimulated PVA dehydrogenase activities of such crude enzyme preparations but not that of the purified membrane-bound PVA oxidase, the following possibilities were proposed for the PQQ effect. (i) The PVA oxidase has PQQ as the coenzyme. The purified PVA oxidase is a holoenzyme. PVA dehydrogenase activities of the crude enzyme preparations were stimulated by PQQ due to the presence of the apoenzyme. (ii) A PVA-dehydrogenating enzyme other than PVA oxidase is present in cells. PVA dehydrogenation by the enzyme in the crude enzyme preparations was stimulated by PQQ.

Determination of the presence of PQQ in membrane-bound PVA oxidase. To examine possibility (i), detection of PQQ in the purified PVA oxidase was attempted with methods similar to those used for the release of PQQ from quinoproteins (4, 5, 14, 16). However, PQQ was not detected in the purified enzyme either treated at 100°C for 30 min in 50 mM KPB (pH 7.5) or hydrolyzed in 6 N HCl at 110°C for 24 h under an N_2 atmosphere. Furthermore, a cell-free extract of VM15C grown on PVA medium supplemented with [^{14}C]PQQ was analyzed by isoelectric focusing (Fig. 2). The peak of PVA oxidase showed a pI of 5.4, coinciding with that of the purified membrane-bound PVA oxidase. The peak of radioactivity was not superimposed on that of PVA oxidase activity, although separation of these peaks was slightly insufficient. Fraction 17, the maximum of the PVA oxidase activity peak, which contained activity of 0.118 U/ml, had radioactivity of only about 115 dpm/ml, which is only slightly higher than the background level. If the enzyme contains an equimolar amount of PQQ as the coenzyme, it can be calculated from the molecular weight and the specific activity of the purified PVA oxidase and the specific radioactivity of [^{14}C]PQQ that radioactivity of 2,790 dpm/ml should be present in the fraction. Thus, the observed radioactivity of the fraction was very low compared with the PVA oxidase activity. These results showed that the membrane-bound PVA oxidase of VM15C does not contain PQQ as the coenzyme.

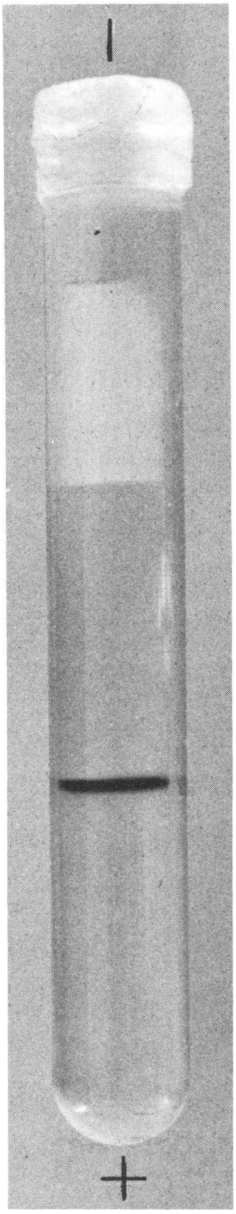

FIG. 1. Disc gel electrophoresis of membrane-bound PVA oxidase. A 20-μg portion of the purified enzyme was subjected to electrophoresis, as described in the text.

PQQ-dependent PVA dehydrogenase. To examine possibility (ii), the solubilized membrane was analyzed by DEAE-Sephacel chromatography with respect to the presence of

TABLE 2. Substrate specificity of membrane-bound PVA oxidase[a]

Substrate	Relative activity (%)
PVA	100
Methanol	0
Ethanol	0
1-Propanol	1.1
1-Butanol	1.3
1-Pentanol	0
1-Hexanol	1.1
1-Heptanol	0
2-Propanol	4.9
2-Pentanol	58.0
2-Hexanol	101.0
4-Heptanol	91.0
2,3-Butanediol	1.0
2,5-Hexanediol	22.0
1,2,6-Hexanetriol	1.4
Hexylene glycol	0

[a] Substrates (0.6 mmol) other than PVA were used in the assay mixture for PVA oxidase activity.

PVA-dehydrogenating enzymes other than PVA oxidase (Fig. 3). PVA dehydrogenase activities of the fractions were assayed after preincubation with and without PQQ. Membrane-bound PVA oxidase was eluted in fractions 4 to 13, in which the PVA dehydrogenase activities were not enhanced by PQQ. On the other hand, although fractions 78 to 94 showed no PVA oxidase activity, and in the case of preincubation without PQQ there was only negligible or slight PVA dehydrogenase activity, these fractions were found to show greatly enhanced PVA dehydrogenase activities in the case of preincubation with PQQ. Thus an enzyme which shows PQQ-dependent PVA dehydrogenase activity was found in the solubilized membrane in addition to the PVA oxidase. The active fractions were collected and the partially purified enzyme was obtained as described above.

Using the preparation, some properties of the enzyme were characterized. The reaction rate as to PVA dehydrogenation by the enzyme was affected by the preincubation time with PQQ (Fig. 4). For the reaction with no preincubation, a marked progressively increasing reaction rate was observed. The maximum and constant reaction rate was attained in the reactions with 30- and 50-min preincubations. The enzyme was noted to require more than 30 min of preincubation with PQQ for full activity. PVA-dehydrogenating activity of the enzyme was examined with several electron acceptors after preincubation with and without PQQ (Table 4). The enzyme showed low activities with PES, PMS, and DCPIP as electron acceptors without PQQ and about 13- to 23-fold enhanced activities with these electron acceptors with PQQ. PVA oxidase activity leading to H_2O_2 formation and PVA-dehydrogenating activities with NAD, NADP, FMN, FAD, and coenzyme Q's were not detected. PVA dehydrogenase activity increased greatly with increasing PQQ used for preincubation at the very low concentration of <20 nM (Fig. 5). Thus, the enzyme was noted to be PQQ-dependent PVA dehydrogenase. The enzyme showed high dehydrogenase activity toward some low-molecular-weight secondary alcohols but not primary alcohols, similarly to the membrane-bound PVA oxidase (Table 5).

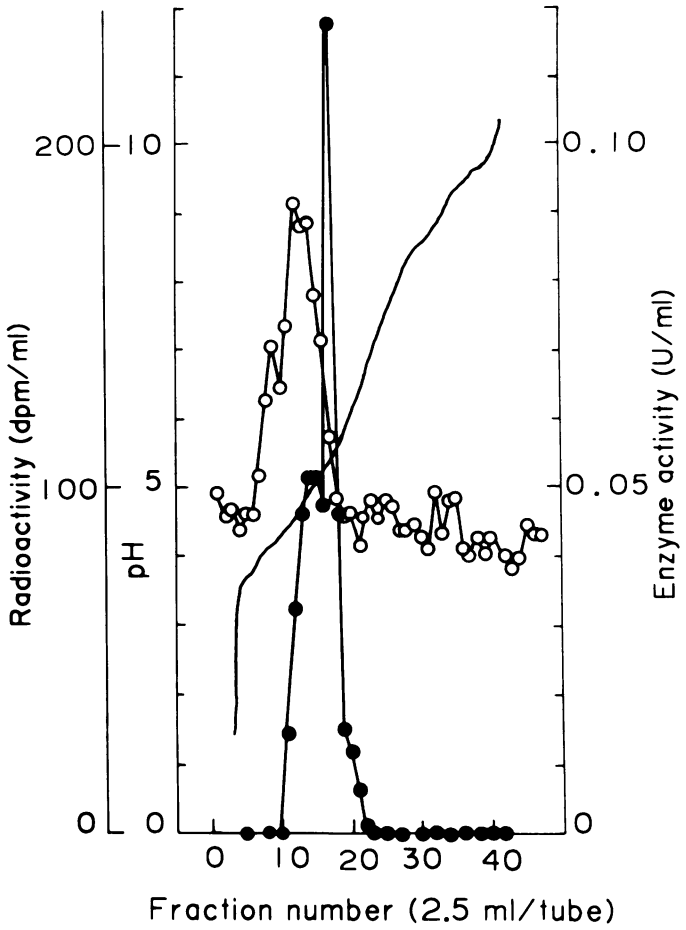

FIG. 2. Isoelectric focusing of a cell-free extract of *Pseudomonas* sp. strain VM15C grown with [^{14}C]PQQ. The cell-free extract (total protein, 130 mg; total radioactivity, 2,110 dpm) prepared from cells grown in PVA medium supplemented with [^{14}C]PQQ at 10 μg/liter for 4 days was solubilized with 2% Triton X-100 for 16 h at 5°C and then subjected to isoelectric focusing after dialysis with 10 mM KPB (pH 7.0) containing 0.1% Triton X-100. Radioactivity (○), PVA oxidase activity (●), and pH (—) of the fractions were measured.

TABLE 3. Effect of PQQ on PVA dehydrogenation by membrane-bound PVA oxidase with various electron acceptors

Electron acceptor[a]	Relative activity (%)	
	−PQQ[b]	+PQQ[c]
O_2[d]	100	100
PES (1.0 mM) + DCPIP (0.1 mM)	330	333
PMS (1.0 mM) + DCPIP (0.1 mM)	342	342
DCPIP (0.1 mM)	22.3	23.0
NAD (1.0 mM)	0	0
NADP (1.0 mM)	0	0
FMN (0.1 mM)	0	0
FAD (0.1 mM)	0	0
Coenzyme Q_6 (0.5 mM)	0	0
Coenzyme Q_{10} (0.5 mM)	0	0

[a] Electron acceptors other than O_2 were used in the assay mixture for PVA dehydrogenase activity at the given concentrations instead of PES and DCPIP.
[b] After preincubation without PQQ for 30 min, the activity was assayed.
[c] After preincubation with 1.36 μM PQQ for 30 min, the activity was assayed.
[d] Activity with O_2 was determined by assaying PVA oxidase activity.

Cytochrome reduction coupled with PVA oxidation by the

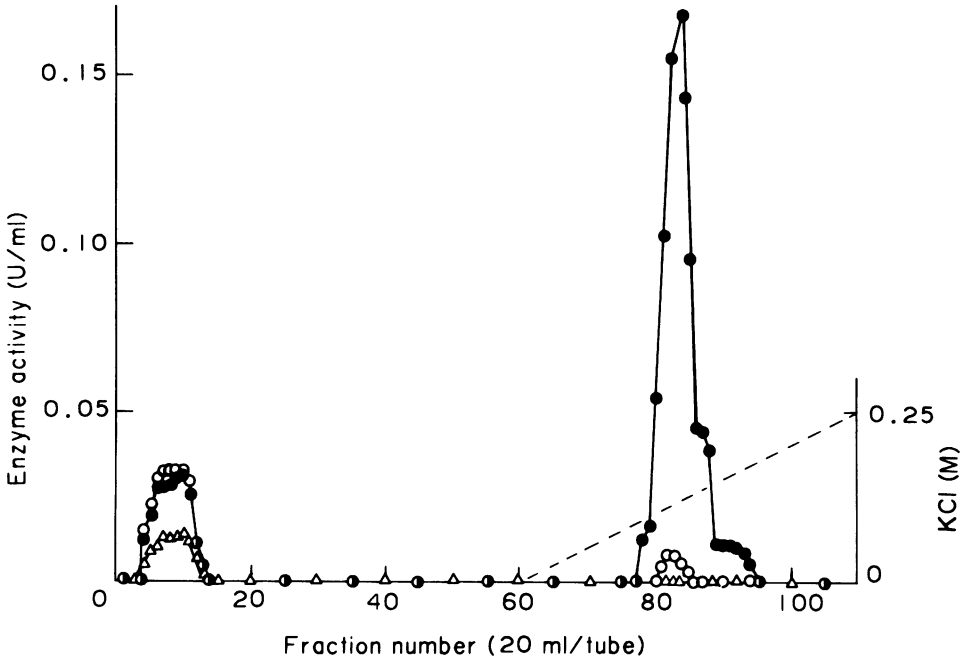

FIG. 3. DEAE-Sephacel chromatography of the solubilized membrane of *Pseudomonas* sp. strain VM15C. Chromatography was performed as described in the text. PVA oxidase activity (△) was determined without preincubation. PVA dehydrogenase activity was assayed after preincubation with 0 (○) and 1.36 (●) μM PQQ for 30 min. The KCl (---) concentration is shown.

membrane fraction. The membrane fraction of VM15C was incubated with PVA, and the difference spectrum was obtained with the "no treatment" one as a reference (Fig. 6). The difference spectrum revealed absorption peaks with maxima at 418, 520, 550, and 600 nm. The peaks at 550, 520, and 418 nm correspond to the α, β, and γ absorption bands of reduced typical type *c* cytochromes (18), respectively. From this, it was suggested that PVA oxidation on the cytoplasmic membrane of VM15C is coupled with the electron transport chain.

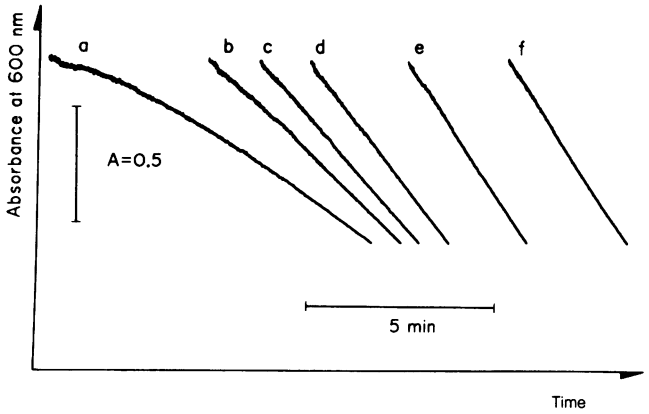

FIG. 4. Effect of preincubation time with PQQ on PQQ-dependent PVA dehydrogenase. The partially purified enzyme (40.8 μg) was preincubated with 1.36 μM PQQ for 0 (a), 5 (b), 10 (c), 15 (d), 30 (e), and 50 (f) min. DCPIP reduction in the assay mixture for PVA dehydrogenase activity was followed at 600 nm with a Shimadzu model UV-200 spectrophotometer.

DISCUSSION

PQQ is an essential growth factor for PVA utilization by *Pseudomonas* sp. strain VM15C and is supplied to the strain by *P. putida* VM15A in PVA-utilizing mixed cultures of the symbionts. In this work, a novel PVA-oxidizing enzyme, PQQ-dependent PVA dehydrogenase, was found in the membrane fraction of VM15C. PQQ is concluded to cause the PVA-dehydrogenating activity of the enzyme in PVA metabolism of the PVA-degrading bacterium through its role as a coenzyme: the symbiosis is related to PVA metabolism through the enzyme.

The activity of the PQQ-dependent PVA dehydrogenase

TABLE 4. Effect of PQQ on PVA dehydrogenation by PQQ-dependent PVA dehydrogenase with various electron acceptors

Electron acceptor[a]	Relative activity (%)	
	−PQQ[b]	+PQQ[c]
O_2[d]	0	0
PES (1.0 mM) + DCPIP (0.1 mM)	4.3	100
PMS (1.0 mM) + DCPIP (0.1 mM)	6.9	119
DCPIP (0.1 mM)	0.9	12.1
NAD (1.0 mM)	0	0
NADP (1.0 mM)	0	0
FMN (0.1 mM)	0	0
FAD (0.1 mM)	0	0
Coenzyme Q_6 (0.5 mM)	0	0
Coenzyme Q_{10} (0.5 mM)	0	0

[a] Electron acceptors other than O_2 were used in the assay mixture for PVA dehydrogenase activity at the given concentrations instead of PES and DCPIP.

[b] After preincubation without PQQ for 30 min, the activity was assayed.

[c] After preincubation with 1.36 μM PQQ for 30 min, the activity was assayed.

[d] Activity with O_2 was determined by assaying PVA oxidase activity.

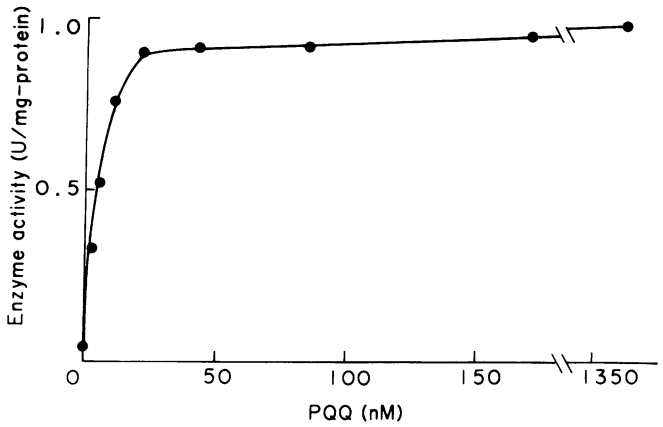

FIG. 5. Effect of PQQ concentration for preincubation on PQQ-dependent PVA dehydrogenase. After preincubation with PQQ at the given concentrations for 30 min, PVA dehydrogenase activity was assayed.

depended on the time and PQQ concentration for preincubation. This dependency coincides with characteristics of typical apo-quinoproteins (5, 33). Quinoproteins have been shown to contain PQQ bound via a covalent or noncovalent bond. The PQQ-dependent PVA dehydrogenase is assumed to be a quinoprotein which binds PQQ via weak noncovalent bonding (holoenzyme) and which easily releases the coenzyme (apoenzyme). The partially purified enzyme was obtained from cells grown in PVA medium supplemented with PQQ, in which the enzyme should be present as a functionally active holoenzyme. The preparation is assumed to contain the enzyme mainly as the apoenzyme, while some PVA dehydrogenase activity, which the preparation showed in the case of preincubation without PQQ, is assumed to be due to the holoenzyme.

The PQQ-dependent PVA dehydrogenase is suggested to be synthesized constitutively by VM15C at least as protein, since all sonic extracts of cells grown under different conditions showed PQQ-dependent PVA dehydrogenase activity.

TABLE 5. Substrate specificity of PQQ-dependent PVA dehydrogenase [a]

Substrate	Realtive activity (%)
PVA	100
Methanol	0
Ethanol	0
1-Propanol	0
1-Butanol	0
1-Pentanol	3.7
1-Hexanol	6.3
1-Heptanol	6.5
2-Propanol	3.3
2-Pentanol	23.4
2-Hexanol	44.0
4-Heptanol	20.4
2,3-Butanediol	0
2,5-Hexanediol	29.5
1,2,6-Hexanetriol	9.8
Hexylene glycol	0

[a] Substrates (0.6 mmol) other than PVA were used in the assay mixture for PVA dehydrogenase activity. The activity was assayed after preincubation with 1.36 μM PQQ for 30 min.

The enzyme would be produced as an apoenzyme in the absence of PQQ by the strain, like the apo-glucose dehydrogenases of *Escherichia coli* (17), *Acinetobacter lwoffi*, and *Pseudomonas aeruginosa* (33) strains.

In addition to the PQQ-dependent PVA dehydrogenase, a membrane-bound PVA oxidase was present in cells of VM15C. The purified enzyme showed about threefold-higher PVA dehydrogenase activity than PVA oxidase activity. The substrate specificity of the enzyme was similar to that of the PQQ-dependent PVA dehydrogenase. However, PQQ did not affect the PVA dehydrogenase activity of the PVA oxidase and the enzyme did not contain PQQ as the coenzyme. Furthermore, the PQQ-dependent PVA dehydrogenase did not show PVA oxidase activity. A VM15C mutant which showed negligible PVA oxidase activity showed relatively high PQQ-dependent PVA dehydrogenase activity (unpublished data). On the basis of these facts, both enzymes are different from each other. However, it may be possible that the PQQ-dependent dehydrogenase is an oligomeric enzyme which has PVA oxidase as a subunit. If this is so, PQQ, as the coenzyme, may not play a direct role in PVA dehydrogenation, since PVA oxidase, which did not contain the coenzyme, itself oxidized PVA with the same electron acceptors as those which the PQQ-dependent PVA dehydrogenase utilized, such as PES, PMS, and DCPIP. PQQ may, in another subunit, participate in secondary electron transfer from the PVA oxidase subunit to the electron acceptors, since the PQQ-dependent dehydrogenase did not utilize O_2, thus differing from PVA oxidase. Despite the possibility of a relationship between the two enzymes, the PQQ-dependent dehydrogenase is indicated to be essential for PVA utilization by VM15C, since the strain

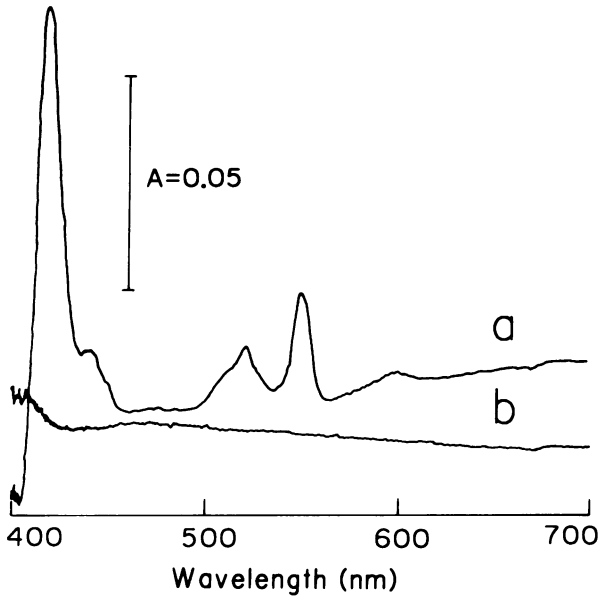

FIG. 6. Difference spectrum of the membrane fraction of *Pseudomonas* sp. strain VM15C after incubation with PVA. The membrane fraction was prepared from cells grown in PVA medium supplemented with PQQ at 10 μg/liter for 4 days. After washing with 50 mM KPB (pH 7.5), the membrane fraction was suspended and incubated in the same buffer at a concentration of 2.82 mg of protein per ml with 0.75% PVA (degree of polymerization = 1,500) for 5 min at room temperature. The difference spectrum (a) and base line (b) were obtained with a Shimadzu model UV-300 spectrophotometer.

required PQQ as an essential growth factor for PVA utilization.

Incubation of the membrane fraction of VM15C with PVA caused a reduction in the cytochrome(s) of the fraction. PVA dehydrogenation on the cytoplasmic membrane is coupled with the electron transport chain. This also shows that PVA dehydrogenation without O_2 as the primary electron acceptor is significant in energy metabolism. The membrane-bound PVA oxidase also showed PVA dehydrogenase activity. Whether PVA dehydrogenation by PVA oxidase or PQQ-dependent PVA dehydrogenase or both can be coupled with the electron transport chain is interesting as to the physiological significance and catalytic roles of the enzymes.

The quinoprotein alcohol dehydrogenases so far reported (2, 8, 13, 16, 19) are more active toward primary alcohols than secondary alcohols. The PQQ-dependent PVA dehydrogenase was active on secondary alcohols rather than primary alcohols. The enzyme is also different from other types of alcohol dehydrogenases (EC 1.1.1.1, 1.1.1.2, etc.). The PQQ-dependent PVA dehydrogenase is concluded to be a novel enzyme, being the first example of a quinoprotein secondary alcohol dehydrogenase, at least in a broad sense, although the function of PQQ as the coenzyme remains to be clarified on the basis of a relationship between the enzyme and PVA oxidase.

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