Three Distinct Quinoprotein Alcohol Dehydrogenases Are Expressed when *Pseudomonas putida* Is Grown on Different Alcohols

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A bacterial strain that can utilize several kinds of alcohols as its sole carbon and energy sources was isolated from soil and tentatively identified as *Pseudomonas putida* HK5. Three distinct dye-linked alcohol dehydrogenases (ADHs), each of which contained the prosthetic group pyrroloquinoline quinone (PQQ), were formed in the soluble fractions of this strain grown on different alcohols. ADH I was formed most abundantly in the cells grown on ethanol and was similar to the quinoprotein ADH reported for *P. putida* (H. Görisch and M. Rupp, Antonie Leeuwenhoek 56:35–45, 1989) except for its isoelectric point. The other two ADHs, ADH IIB and ADH IIG, were formed separately in the cells grown on 1-butanol and 1,2-propanediol, respectively. Both of these enzymes contained heme *c* in addition to PQQ and functioned as quinohemoprotein dehydrogenases. Potassium ferricyanide was an available electron acceptor for ADHs IIB and IIG but not for ADH I. The molecular weights were estimated to be 69,000 for ADH IIB and 72,000 for ADH IIG, and both enzymes were shown to be monomers. Antibodies raised against each of the purified ADHs could distinguish the ADHs from one another. Immunoblot analysis showed that ADH I was detected in cells grown on each alcohol tested, but ethanol was the most effective inducer. ADH IIB was formed in the cells grown on alcohols of medium chain length and also on 1,3-butanediol. Induction of ADH IIG was restricted to 1,2-propanediol or glycerol, of which the former alcohol was more effective. These results from immunoblot analysis correlated well with the substrate specificities of the respective enzymes. Thus, three distinct quinoprotein ADHs were shown to be synthesized by a single bacterium under different growth conditions.

Alcohol dehydrogenases (ADHs) whose reaction is independent of NAD(P) have been found in many aerobic bacteria. Most of these enzymes contain pyrroloquinoline quinone (PQQ) as a prosthetic group and are termed quinoprotein ADHs. One example is methanol dehydrogenase, which is present in methylotrophic bacteria (6). ADHs other than methanol dehydrogenase, found in nonmethylotrophic bacteria, have been classified into three groups (types I through III) according to their molecular properties (17). The type I ADH has been purified from *Pseudomonas aeruginosa* (11, 18, 19) and *Pseudomonas putida* (10). Its molecular structure resembles that of methanol dehydrogenase in many respects, but it has a low affinity for methanol. The type III ADH is a membrane-bound enzyme found in the cytoplasmic membrane of acetic acid bacteria (1, 2, 17). It is a complex of three different subunits, quinohemoprotein ADH (subunit I), cytochrome *c* (subunit II), and a third small subunit (subunit III) whose function is unknown. Subunit I contains 1 mol each of noncovalently bound PQQ and covalently bound heme *c* as the prosthetic group in a single peptide and has a molecular mass of 70 to 80 kDa. Complete separation of subunit I from the other components has not been successful, since such a membrane-bound enzyme becomes inactivated and aggregated because of hydrophobicity when it is treated in the absence of detergents. If subunit I can be separated stably or if the subunit I-like ADH is found in nature, some basic information relating to the reaction mechanism of the type-III ADH will become available. Thus, finding the subunit I-like ADH in the soluble fraction of aerobic bacteria has been much anticipated.

A quinohemoprotein ADH from *Comamonas testosteroni* (12) which occurs in the soluble fraction as a single protein and resembles subunit I in the type III ADH, although the enzyme has been purified as an apoenzyme, has been assigned to the type II ADHs. Since *C. testosteroni* cannot produce PQQ, the enzyme activity appears only after exogenous addition of PQQ (12). In order to find a microorganism that produces the type II ADH holoenzyme, bacteria were screened for growth on various kinds of alcohols.

A bacterial strain that could utilize several different kinds of alcohol as its sole carbon and energy sources was isolated from soil. The bacterium produced three different dye-linked ADHs containing PQQ, all of which were purified in this study. One is similar to the type I ADH reported for *P. putida* (10). The other two enzymes belong to the type II ADHs; they are formed in 1-butanol- or glycerol-grown cells and have been designated ADH IIB and ADH IIG, respectively. The latter enzyme is induced much more effectively with 1,2-propanediol and can react with primary alcohols as well as with glycerol. This is the first report that three different quinoprotein ADHs are synthesized in a single microorganism.

MATERIALS AND METHODS

Chemicals. All chemicals used in this study were commercial products of guaranteed grade. Yeast extract was a product of the Oriental Yeast Co. Special care was paid to remove traces of alcohol in chemicals which came from alcohols used as dehydrants in chemical manufacturing. For the preparations of buffer solutions and ingredients used for measurements of ADH activity, such contaminating alcohol was removed by evaporation at 80°C under reduced pressure.

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Growth medium and bacterial cultivation. Basal medium used for bacterial isolation and cultivation was composed of the following ingredients (per liter): 2 g of NaNO$_3$, 2 g of (NH$_4$)$_2$SO$_4$, 2 g of K$_2$HPO$_4$, 1 g of KH$_2$PO$_4$, 0.2 g of MgSO$_4 \cdot$ 7H$_2$O, and 0.5 g of yeast extract. These ingredients were dissolved in tap water, and the pH was adjusted to 7.0 before sterilization at 120°C for 20 min. The carbon source used was added separately to a final concentration of 0.2%. Cells were grown at 30°C on a rotary shaker in 100 ml of the basal medium to early stationary phase. The bacterial growth was monitored with a Klett-Summerson colorimeter.

Preparation of soluble fractions. All procedures for preparation of soluble fractions and further purification of the three ADHs were performed at 4°C unless otherwise stated. Cells were harvested, washed once with ice-cold saline, and suspended in 5 ml of 50 mM Tris-HCl buffer (pH 8.0). The suspension was passed through a French pressure cell at 16,000 lb/in^2 at 4°C. After removal of intact cells, the cell homogenate was centrifuged at 100,000 $\times$ g for 90 min, and the resulting supernatant was obtained as a soluble fraction.

Enzyme assays. All enzyme assays were performed at 25°C as follows. The enzyme activity of NAD-dependent ADH was measured by the increase in A_{340} of NADH in a reaction mixture (total, 3 ml) consisting of 50 mM Tris-HCl (pH 8.0), 2 mM NAD, and enzyme solution. The reaction was started by adding 1-butanol at a final concentration of 10 mM. The enzyme activity observed with substrate was subtracted from that obtained without substrate. Phenazine methosulfate (PMS) reductase activity was measured spectrophotometrically by monitoring the reduction of 2,6-dichlorophenol indophenol (DCIP) at 600 nm in a reaction mixture consisting of 50 mM Tris-HCl (pH 9.0), 0.4 mM PMS, 0.22 mM DCIP, 50 mM ethylamine, and 10 mM substrate in a total volume of 3 ml. Ferricyanide reductase activity was assayed as described previously (3) in 50 mM Tris-HCl (pH 8.0) with 10 mM substrate. One unit of the enzyme activity was defined as the amount of enzyme catalyzing oxidation of 1 μmol of substrate per minute under the conditions described above. Protein content was estimated by a modified method of Lowry (9) with bovine serum albumin as a standard.

Purification of ADH I. The soluble fraction was prepared from cells (30 g of wet cells) grown on ethanol as described above except that 50 mM Tris-HCl (pH 7.9) containing 10 mM CaCl$_2$ was used. The soluble fraction was applied to a DEAE-cellulose column (5 by 18 cm) preequilibrated with the same buffer. The enzyme was not adsorbed to the column and eluted with the same buffer. Active fraction thus collected was applied to a phenyl-Sepharose column (1.8 by 20 cm) equilibrated with the same buffer. Enzyme activity was eluted with 25 mM Tris-HCl (pH 7.9) containing 5 mM CaCl$_2$ and 50% ethylene glycol. The eluted enzyme fractions were collected and then concentrated by ultrafiltration. The concentrated enzyme was subjected to gel filtration with a Sephacryl S-200 column (1.6 by 90 cm) preequilibrated with 50 mM Tris-HCl (pH 8.0) containing 10 mM CaCl$_2$ and 0.15 M KCl. After being eluted with the same buffer, the active fractions were pooled, dialyzed against 50 mM Tris-HCl (pH 8.0) containing 10 mM CaCl$_2$, and used as the purified enzyme.

Purification of ADH IIB. The soluble fraction prepared from 150 g of 1-butanol-grown cells was put on a DEAE-cellulose column (3 by 30 cm) which had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The enzyme was not adsorbed with the column. The unadsorbed fractions were combined, and the enzyme was precipitated by adding polyethylene glycol 6000 (PEG 6000) to 20% (wt/vol). Then the collected precipitate was dissolved in 10 mM sodium acetate buffer (pH 6.0) and dialyzed overnight against the same buffer. After elimination of insoluble materials by conventional centrifugation, the enzyme solution was put on a CM-Sephadex C-50 column (2 by 20 cm) which had been equilibrated with 10 mM sodium acetate buffer (pH 6.0). The enzyme was eluted with the same buffer after a major protein peak of unadsorbed fractions eluted, since it seemed to have a loose interaction with CM-Sephadex under the conditions used. Fractions having enzyme activity were combined and PEG 6000 was added as described above. The precipitate collected was dissolved in a minimal volume of 50 mM Tris-HCl (pH 8.0) and further purified with a Sephadex G-200 gel column (1 by 180 cm) which had been equilibrated with the same buffer. The active fractions were combined and used as the purified enzyme.

Purification of ADH IIG. The soluble fraction was obtained from 1,2-propanediol-grown cells (100 g [wet weight]) as described above except that pH 7.0 instead of pH 8.0 buffer was used. The fraction was applied to a DEAE-cellulose column (5.1 by 20 cm) preequilibrated with 50 mM Tris-HCl buffer (pH 7.0). Because of the presence of impurities which interact with ADH IIG rather than DEAE-cellulose, the enzyme was not adsorbed into the column and came out after a main peak of impurities. Then the active fractions were collected and applied again to the same column regenerated under the same conditions. The enzyme activity was adsorbed and eluted with the same buffer containing 100 mM KCl. The active fractions were collected, concentrated with PEG 6000, and dialyzed against 10 mM Tris-HCl buffer (pH 8.5) containing 10 mM CaCl$_2$. The dialyzed enzyme was applied to a DEAE-Toyopearl column (2.6 by 16.5 cm) pretreated with the same buffer. The enzyme activity was eluted with a linear gradient of KCl up to 50 mM in the same buffer. The active fractions were concentrated with PEG 6000 and dialyzed against 100 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl and 10 mM CaCl$_2$. The dialyzed enzyme was subjected to gel filtration on a Sephacryl S-200 column (1.6 by 90 cm), and the enzyme was eluted with the same buffer. The active fractions were pooled and used as the purified enzyme.

Analytical ultracentrifugation. Estimation of the purity and measurement of the sedimentation velocity of the purified enzyme were done with a Hitachi model SCP85H ultracentrifuge at 60,000 rpm with Schlieren optics and adsorption scanning, respectively.

Analytical gel filtration. An alternative molecular weight determination was performed by gel filtration (column, 1 by 30 cm) with Superdex 200 HR 10/30 (Pharmacia). Elution was done at a flow rate of 0.5 ml/min with 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl and was monitored at 280 nm. The following marker proteins (Oriental Yeast Co.) with the indicated M_rs were used for calibration: yeast glutamate dehydrogenase, 290,000; porcine heart NAD-lactate dehydrogenase, 142,000; yeast enolase, 67,000; yeast adenylate kinase, 32,000; and horse heart lysozyme, 12,400.

PAGE. Polyacrylamide gel electrophoresis (PAGE) in the absence of sodium dodecyl sulfate (SDS) was done with an 8% polyacrylamide gel and Tris-glycine buffer (pH 8.3). Nondenaturated ADHs were detected with their enzyme activities by soaking the developed gel in the same reaction mixture as for the PMS-DCIP assay except containing a few crystals of nitroblue tetrazolium instead of DCIP. SDS-PAGE was done with a slab gel as described by Laemmli (15). After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250. The marker proteins used for estimation of molecular weight were phosphorylate b (M_r, 92,000), bovine serum albumin (M_r, 68,000), carbonic anhydrase (M_r, 31,000), soybean trypsin inhibitor (M_r, 21,500), and lysozyme (M_r, 14,000).

Isoelectric focusing. Isoelectric focusing was performed with a 5% polyacrylamide gel containing 5% Ampholine (pH 3.5 to 10) (Pharmacia). Electrophoresis was carried out at 200 V for 4 h with 0.02 M phosphoric acid as the anode solution and 1 M NaOH as the cathode solution. Cytochrome c (pI 10.6) and acetylated cytochromes c (pI 9.7, 8.3, 6.4, 4.9, and 4.1) were used as pI markers (Oriental Yeast Co.). The gels were soaked with 3.5% (wt/vol) perchloric acid, and then the proteins were stained with Coomassie brilliant blue G-250 in 3.5% perchloric acid.

Preparation of antibodies. Three different antibodies for ADHs IIB and IIG purified from *P. putida* HK5 and for ADH from *P. aeruginosa* (19) were prepared in this study. The enzymes (1 mg each) were injected separately into different rabbits with Freund's complete adjuvant. One month later, a booster injection of 0.5 mg of protein mixed with Freund's incomplete adjuvant was administered. Another 2 weeks later, blood was collected and left at 4°C overnight. After centrifugation to remove the erythrocytes, the antisera were obtained and stored at −80°C.

Immunoblot analysis. The proteins in the gel after SDS-PAGE were transferred electrophoretically onto a polyvinylidene difluoride membrane at 50 mA overnight. The membrane was blocked with 3% gelatin, washed with Tris-buffered saline (TBS) (20 mM Tris-HCl, 500 mM NaCl; pH 7.5), and then incubated with TBS containing antibody for 2 h. The membrane was washed again and incubated with TBS containing protein A-peroxidase conjugate for 2 h. After the membrane was washed with TBS containing 0.05% Tween 20, the enzyme band was visualized by the addition of color reagents and H$_2$O$_2$. The prestained marker proteins (Bio-Rad) used for estimation of relative mobility were phosphorylase b (M_r, 142,900), bovine serum albumin (M_r, 97,200), ovalbumin (M_r, 50,000), carbonic anhydrase (M_r, 35,100), soybean trypsin inhibitor (M_r, 29,700), and lysozyme (M_r, 21,900).

Measurement of heme c content. The amount of heme c in the purified enzyme was measured from the reduced minus oxidized difference spectra of its pyridine hemochrome, using a millimolar extinction coefficient of 24.3 (549 nm − 535 nm). The pyridine hemochrome was prepared by adding pyridine to 20% and then NaOH to 0.2 N final concentrations to the enzyme solution under essentially the same conditions as described previously (16).

Measurement of PQQ content. PQQ in the purified enzyme was extracted with methanol and measured with apo-glucose dehydrogenase as described previously (4).

Type strains. Type strains of the genus *Pseudomonas* used as reference strains in identification of the isolated bacterium were donations from the Institute for Fermentation, Osaka (IFO), Japan.

RESULTS

Taxonomic characterization of the isolated microorganism. A bacterial strain was isolated from soil as a 1-octanol utilizer. The strain was tentatively identified as *P. putida* on the basis of physiological and biochemical properties as listed in Table 1. However, this strain was unusual in that it did not produce pigment in King's B medium and could not utilize benzylamine as a carbon source. Six strains of *Pseudomonas* were employed as reference strains: *P. putida* IFO 3738, *P. putida* IFO 12653, *P. aeruginosa* IFO 3445, *P. aeruginosa* IFO 3923, *P. aeruginosa* IFO 3924, and *P. fluorescens* IFO 3081. All of these strains were not able to grow on 1-octanol as the sole carbon source in

TABLE 1. Physiological and biochemical characteristics of the isolated microorganism

Test	Result
Bacteriological	
Gram stain	−
	KOH test +
Spores	−
Motility	+
Morphology	Short rods
Fermentation in glucose OF[a]	−
UV fluorescence	+
Pigment production in King's B medium	−
Growth at:	
37°C	+
41°C	−
45°C	−
Growth	
D-Glucose	+
D-Xylose	−
D-Galactose	−
Arabinose	−
Mannose	−
Mannitol	−
N-Acetylglucosamine	−
Maltose	−
Lactose	−
Sucrose	−
Trehalose	−
Gluconate	+
Caprate	+
Adipate	−
Succinate	+
Malate	+
Citrate	+
Phenylacetate	+
Inositol	−
Benzylamine	−
Biochemical	
Catalase	+
Oxidase	+
Oxidation of gluconate	+
2-Ketogluconate production	−
Nitrate reduction	−
Indole production	−
Acid from glucose	−
Arginine dihydrolase	+
Urease	−
Esculin hydrolysis	−
Gelatin hydrolysis	−
β-Galactosidase	−
Cytochrome oxidase	+
Acid from maltose	−
Growth in 5% NaCl	+
Egg yolk agar opacity	−
Levan from sucrose	−
Reaction in litmus milk	Alkaline
Hydrolysis of gelatin	−
Hydrolysis of starch	−

[a] OF, oxidation-fermentation.

the liquid medium used in this study. Thus, the new strain was tentatively identified as *P. putida* HK5.

P. putida HK5 could utilize all of the aliphatic primary alcohols examined except for methanol and 1-heptanol. 1-Hexanol and 1-octanol were poor substrates for growth. On the basis of the turbidity of the liquid culture, 1-nonanol, 1-decanol, and 1-dodecanol appeared to be better growth substrates than 1-octanol, although some biosurfactant seemed to be produced on the three former substrates. Some diols, such as 1,2-propanediol and 1,3-propanediol, and glycerol were also utilized by the organism, but several sugar alcohols, such as D-mannitol and D-sorbitol, were not.

This organism could also utilize D-glucose as the sole carbon source but not D-xylose, D-galactose, maltose, lactose, or sucrose. Some organic acids, such as acetate, succinate, citrate, and butyrate, were able to be used as carbon sources, while butylamine and other monoamines examined were not utilized.

Enzyme activities of ADHs in the soluble fraction. ADH activities of the soluble fractions from the cells grown on different alcohols were measured (Table 2). No significant ADH activity was seen in the membrane fraction (data not shown). As shown in Table 2, in alcohol-grown cells, NAD-dependent ADH activity was much lower than PMS reductase activity when both enzyme activities were measured with 1-butanol used as a substrate. Dye-linked ADH from *P. putida* has been reported to be almost inactive in the absence of exogenous ammonium ions or amines (10). However, 1-butanol- or 1,2-propanediol-grown cells showed relatively high levels of PMS reductase activity in the absence of exogenous ammonium ions or amines.

Ferricyanide reductase activity in the soluble fraction was assayed with 1-butanol and glycerol as substrates (Table 2). Since ADH I cannot reduce ferricyanide (see below), the ferricyanide reductase activities observed come from the presence of ADHs IIB and IIG. Glycerol can be used as a substrate to distinguish ADH IIG from ADH IIB because ADH IIB did not react with glycerol (see below). When the bacterial strain was grown on 1-butanol, 1,2-propanediol, or glycerol, ferricyanide reductase activity with 1-butanol was observed. With glycerol as a substrate, only 1,2-propanediol- and glycerol-grown cells showed ferricyanide reductase activity. When the bacterial strain was grown on D-glucose or Luria-Bertani medium, no significant enzyme activities of the three PQQ ADHs were detected.

Electrophoretic identification of the three ADHs in the soluble fraction. ADH activity-dependent staining of electrophoretic gels containing the soluble fractions of cells grown on different alcohols was performed (Fig. 1). A total of three ADHs with different mobilities were detected in the soluble fractions from cells grown on ethanol, 1-butanol, or 1,2-propanediol. The enzyme activity band with the lowest mobility showed the strongest activity in the presence of ethylamine (Fig. 1B) and was seen in cells grown on all three alcohols; this band was designated ADH I. The middle band was stained with 1-butanol as a substrate in the absence of ethylamine (Fig. 1C). This band was seen in both 1-butanol-grown and 1,2-propanediol-grown cells, but the intensity was weak in the latter preparation; this band was designated ADH IIB. The enzyme activity band with the highest mobility was detected only in 1,2-propanediol-grown cells (Fig. 1D). This band was stained with glycerol as a substrate and, to a lesser extent, with 1-butanol. In both cases, no ethylamine was present. This band was designated ADH IIG. All three activity bands were hardly detectable in cells grown on D-glucose and Luria-Bertani medium (data not shown), although a faint ADH I band was occasionally found in different batches of D-glucose-grown cells. These data showed a good correlation with those in Table 2.

Purification and properties of ADH I. ADH I, whose activity is greatly enhanced in the presence of ethylamine, was purified from the soluble fraction of ethanol-grown cells (Table 3). The purified enzyme showed a single band in both SDS-PAGE (Fig. 2A) and PAGE without SDS (Fig. 2C). The former gel indicated that the enzyme has a molecular weight of 64,000. By analytical gel filtration, the molecular weight was estimated to

TABLE 2. ADH activities in soluble fractions from cells grown on different carbon sources

Growth medium	NAD-ADH, 1-butanol	PMS reductase		Ferricyanide reductase	
		1-Butanol	Butanol + amine[b]	1-Butanol	Glycerol
Luria-Bertani	5.1	ND[c]	ND	ND	ND
Basal plus:					
D-Glucose	1.3	ND	ND	ND	ND
Ethanol	ND	12.5	460	ND	ND
1-Butanol	2.7	300	482	234	ND
1,2-Propanediol	2.8	42.0	380	234	312
Glycerol	2.7	19.4	62.9	105	104

Sp act (mU/mg) of enzyme with the indicated alcohol[a]

[a] All enzyme activities were measured in 50 mM Tris-HCl (pH 8.0). Substrates were used at a concentration of 10 mM. Data are means of three independent experiments.
[b] Ethylamine (50 mM) was added as an activator.
[c] ND, not detected.

be 105,000. Schrover et al. (19) have reported that ADH from *P. aeruginosa* contains a small additional subunit with an $\alpha_2\beta_2$ structure. However, this small subunit was not found by SDS-PAGE (Fig. 2A) or gel filtration which was performed in the presence of SDS and monitored for A_{214} (data not shown). Thus, ADH I is believed to consist of two identical subunits. The enzyme contained 2.2 mol of PQQ per mol of enzyme on the basis of the molecular weight of 130,000. ADH I reacted well with PMS as an electron acceptor but not with NAD(P), potassium ferricyanide, or molecular oxygen. Little enzyme activity was detected in the absence of ethylamine or ammonium ions. The optimum pH in the enzyme reaction mixture was around 9.0. The absorption spectrum showed absorption maxima at 278 and 340 nm (Fig. 3A), which were almost the same as those reported for the quinoprotein ADH from *P. aeruginosa* (11). Treatment of ADH I with EDTA allowed dissociation of the enzyme into two identical monomer subunits, releasing PQQ. Thus, PQQ was involved noncovalently with ADH I. In Fig. 3A, the absorption spectrum of ADH I apoenzyme is shown. The enzyme cross-reacted well with the antibody raised against quinoprotein ADH from *P. aeruginosa* (see below). The isoelectric point of this enzyme was determined to be 7.6 by isoelectric focusing. This is lower than the pI values of 9.1 and 8.2 that were reported for quinoprotein ADHs from *P. putida* and *P. aeruginosa*, respectively (10).
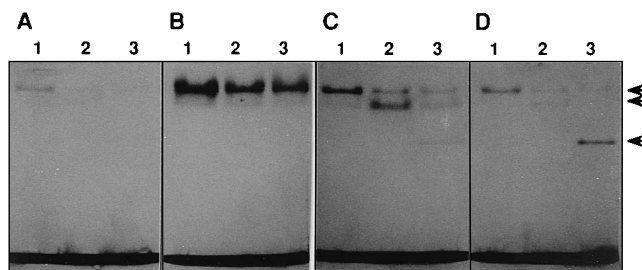

FIG. 1. Enzyme activity staining of the three distinct ADHs in the soluble fractions. Gels after PAGE without SDS were stained with PMS and nitroblue tetrazolium. Either no substrate (A) or 1 mM ethanol and 50 mM ethylamine (B), 1 mM 1-butanol (C), or 10 mM glycerol (D) was used. The soluble fractions (100 μg of protein) used were from cells grown on ethanol, 1-butanol, and 1,2-propanediol (lanes 1 to 3, respectively). Bands corresponding to ADHs I, IIB, and IIG are indicated (arrows, from top to bottom, respectively). The thick line visible in the bottom of the gel is bromophenol blue. A trace of contaminated alcohol in buffer solution had been eliminated as described in Materials and Methods.

Purification and properties of ADHs IIB and IIG. The other two ADHs, IIB and IIG, were purified from the soluble fractions of the cells grown on 1-butanol and 1,2-propanediol, respectively (Fig. 2C). Although ADH IIG had originally been purified from glycerol-grown cells, cells grown on 1,2-propanediol were used in this study because they were found to synthesize ADH IIG more effectively (see Fig. 5). Potassium ferricyanide was used as an electron acceptor in routine assays for both ADH IIB and ADH IIG because both enzymes reacted well with it but ADH I did not. The purifications of the enzymes are summarized in Tables 4 and 5 for ADHs IIB and IIG, respectively. The optimum pH for both enzymes was around 8.0. Both enzymes could utilize PMS as an electron acceptor, but it was much less reactive than potassium ferricyanide in ADH IIG. NAD(P) and molecular oxygen were inert for both enzymes.

The molecular weights of ADHs IIB and IIG were estimated to be 63,000 and 68,000 by gel filtration chromatography and to be 69,000 and 72,000 by SDS-PAGE, respectively (Fig. 2B), indicating that both enzymes exist as monomers. In analytical ultracentrifugation, a single sedimentation peak was observed with $s_{20,w}$ values of 4.0 and 4.6 s for ADHs IIB and IIG, respectively. These were measured with various enzyme concentrations. Like the results of SDS-PAGE and gel filtration, these values suggest that the enzymes exist as monomers. ADHs IIB and IIG contained, respectively, 1.4 and 1.2 mol of PQQ per mol of enzyme, on the basis of a molecular weight of 70,000. PQQ was released identically from both enzymes by EDTA treatment or heat denaturation, indicating noncovalent binding of PQQ to the enzymes. The isoelectric points of ADHs IIB and IIG were determined to be 6.5 and 5.0, respectively.

Both enzymes showed a rose-red color and a typical reduced

TABLE 3. Summary of purification of ADH I[a]

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)
Soluble fraction	2,280	3,080	1.35	100
DEAE-cellulose	451	4,560	10.1	148
Phenyl-Sepharose	132	3,430	26.1	111
Gel filtration	101	2,700	26.8	88

[a] Enzyme activity was measured in 50 mM glycine-NaOH buffer (pH 9.0) containing 0.4 mM PMS, 0.22 mM DCIP, 50 mM ethylamine, and 10 mM ethanol.
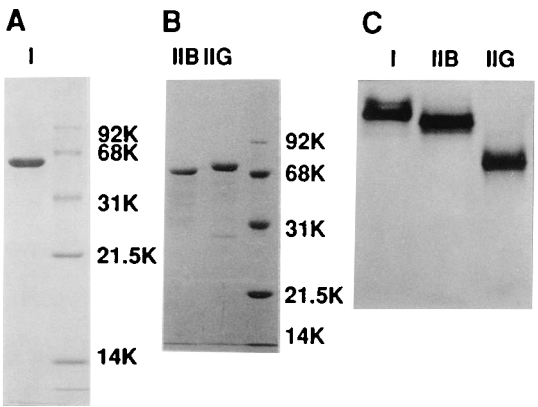
FIG. 2. Electrophoretic properties of the three ADHs. After electrophoresis, proteins were stained with Coomassie brilliant blue R-250. (A and B) SDS-PAGE. The concentrations of acrylamide in the separation gels were 12.5 and 10%, respectively. Purified enzymes (2 μg of each) were loaded. (C) PAGE without SDS. Purified enzymes (20 μg of each protein) were loaded. Molecular weights are indicated in thousands.

TABLE 4. Summary of purification of ADH IIB[a]

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)
Soluble fraction	15,150	1,160	0.070	100
DEAE-cellulose	3,520	1,010	0.280	87
CM-Sephadex	260	810	3.14	70
Gel filtration	65	490	7.63	42

[a] Enzyme activity was measured in 50 mM Tris-HCl (pH 8.0) containing 20 mM potassium ferricyanide and 10 mM 1-butanol.

form of cytochrome *c*-type spectrum. ADH IIB had absorption peaks at 278, 418, 523, and 550 nm (Fig. 3B), and peaks at 278, 415, 523, and 552 nm were seen for ADH IIG (Fig. 3C). When ADHs IIB and IIG were oxidized with potassium ferricyanide, the peaks around 520 to 560 nm were diminished and the peaks at 418 or 415 nm were shifted to 410 nm. Because of the presence of excess potassium ferricyanide, however, it was

difficult to measure the absorption spectra of the oxidized enzymes absolutely. The cytochromes *c* in both enzymes tend to exist in reduced form, as seen with the type III ADH from acetic acid bacteria (1, 2, 17). When treated with 20 mM EDTA and 1.5 M urea, both enzymes were converted into apoenzymes, releasing PQQ. In both apoenzymes, cytochrome *c* existed in oxidized form, of which the γ-band was shifted to around 410 nm, as shown in Fig. 3B and C. From the difference spectra of pyridine hemochrome, ADHs IIB and IIG were calculated to contain 1.0 and 1.2 mol of heme *c* per mol of the enzyme, respectively, on the basis of a molecular weight of 70,000. The heme *c* was bound covalently to both enzymes.

Substrate specificities of the three ADHs. The substrate specificities of the three ADHs purified in this study are shown in Table 6. ADH I showed a broad specificity toward various primary alcohols, of which ethanol was the best substrate. ADH IIB could react with primary and secondary alcohols. It
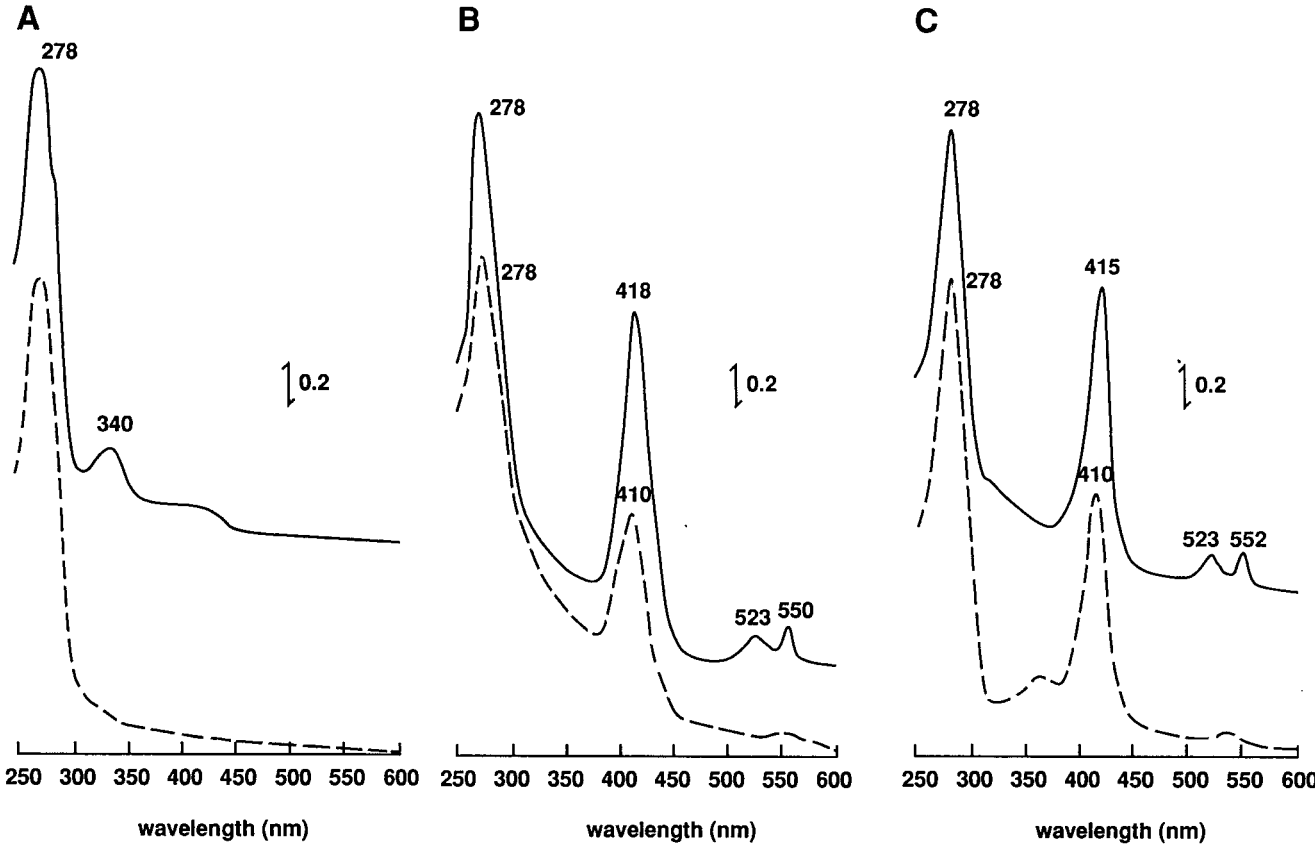

FIG. 3. Absorption spectra of the three purified ADHs measured in 50 mM Tris-HCl (pH 8.0). (A) ADH I (1.7 mg/ml); (B) ADH IIB (1.08 mg/ml); (C) ADH IIG (1.13 mg/ml). Results for the purified ADHs (solid lines) and the respective apoenzymes (broken lines) are shown. All spectra were measured at room temperature.

TABLE 5. Summary of purification of ADH IIG[a]

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)
Soluble fraction	7,170	329	0.0460	
DEAE-cellulose				
Passage	2,660	719	0.270	100
Adsorption	106	518	4.89	72
DEAE-Toyopearl	37.2	439	11.8	61
Gel filtration	19.7	275	14.0	38

[a] Enzyme activity was measured in 50 mM Tris-HCl (pH 8.0) containing 20 mM potassium ferricyanide and 10 mM glycerol.

showed a preference for C_4 to C_7 alcohols but never reacted with methanol or glycerol. Enzyme activity of ADH IIG was observed with primary alcohols as well as glycerol but not methanol. Among the substrates examined, ADH IIG could react with 1,2-propanediol most efficiently under the assay conditions used. The three enzymes could react with 1,2-propanediol and 1,3-propanediol but not with several sugar alcohols, such as D-sorbitol. The following substrates were inert for all three enzymes: tert-amyl alcohol, 2-methyl-2-propanol, xylitol, adonitol, D-sorbitol, D-mannitol, L-dulcitol, D-lactate, D-glucose, D-fructose, PEG 6000, and polyvinyl alcohol. Consequently, it seems that ADH I prefers shorter monoalcohols, ADH IIB prefers longer monoalcohols, and ADH IIG prefers vicinal diols but not sugar alcohols.

Apparent kinetic constants of the three enzymes with ethanol, 1-butanol, 1,2-propanediol, and glycerol were measured (Table 7). ADH I showed a higher affinity for ethanol than 1-butanol. ADHs IIB and IIG showed similar apparent K_m and V_{max} values with 1-butanol. ADH IIG showed a higher affinity for 1-butanol than for 1,2-propanediol. At a high concentration of 1-butanol (10 mM), enzyme activity was repressed in both enzymes (data not shown).

Immunoblot analysis of the three ADHs. Immunoblot analysis was performed with the antisera raised separately against the purified ADHs IIB and IIG and against ADH from *P. aeruginosa*. ADH I reacted well with the antibodies raised against ADH from *P. aeruginosa*, and each of the three antibodies was shown to be specific for one of the three ADHs (Fig. 4). None of these antibodies reacted with ADHs from *C. testosteroni*, *Gluconobacter suboxydans*, or *Acetobacter aceti* (data not shown).

To determine precisely which ADH was synthesized by which growth substrate, immunoblot analysis with the different soluble fractions was performed (Fig. 5). In each case, a high-intensity band which cross-reacted specifically with the respective antiserum was detected, although some minor bands with lower intensities were also detected. ADH I was found in all soluble fractions examined and was most abundant in ethanol-grown cells (Fig. 5A). A faint band cross-reacting with ADH I antibody was found in cells grown on D-glucose, although no significant enzyme activity was detected in these cells, as shown in Table 2. ADH IIB was found in cells grown on 1-propanol, 1-butanol, 1,2-propanediol, or 1,3-butanediol but was hardly detectable in cells grown on ethanol (Fig. 5B). 1-Octanol, which was used originally as the growth substrate for bacterial screening, produced only small amounts of ADHs I and IIB, which is consistent with the poor bacterial growth observed as described above. ADH IIG was found only in cells grown on 1,2-propanediol or glycerol and was more abundant in the former preparation (Fig. 5C). These results correlate well with the substrate specificities of the enzymes (Table 7).

TABLE 6. Substrate specificities of the three ADHs

Substrate[a]	Relative activity (%)[b]		
	ADH I	ADH IIB	ADH IIG
Primary alcohols			
Ethanol	100	78	22
1-Propanol	103	70	41
1-Butanol	87	100	42
1-Pentanol	113	100	40
1-Hexanol	109	70	46
1-Heptanol	99	99	39
1-Octanol	73	44	24
Allyl alcohol	124	160	46
iso-Amyl alcohol	54	100	93
Secondary alcohols			
2-Propanol	31	52	0
2-Butanol	48	90	15
2-Pentanol	4	120	19
2-Hexanol	3	99	33
3-Pentanol	6	43	15
Diols			
1,2-Ethanediol	18	0	71
1,2-Propanediol	7	43	100
1,3-Propanediol	13	61	42
1,2-Butanediol	2	84	86
1,3-Butanediol	1	105	75
1,4-Butanediol	8	110	75
2,3-Butanediol	0	12	71
2,4-Pentanediol	8	45	0
Sugar alcohols			
Glycerol	0	0	63
meso-Erythritol	0	0	10
Aldehydes			
Acetaldehyde	41	82	16
Propionaldehyde	66	125	58
Butyraldehyde	26	32	13
Glyceraldehyde			58
Others			
Ethyleneglycol ethyl ether	81	36	23
2-Methyl-2,4-pentanediol	0	21	0

[a] The final concentration of each substrate used in the reaction mixture was 10 mM. The following substrates were inert: methanol, tert-amyl alcohol, 2-methyl-2-propanol, xylitol, adonitol, D-sorbitol, D-mannitol, L-dulcitol, D-lactate, D-glucose, D-fructose, PEG 6000, and polyvinyl alcohol.
[b] Enzyme activities were measured as described in Materials and Methods. The enzyme activity with each substrate was expressed relative to a value of 100 for the activity of ADH I with ethanol, ADH II with 1-butanol, or ADH IIG with 1,2-propanediol.

DISCUSSION

A bacterial strain originally isolated as a 1-octanol utilizer was shown to grow on many different kinds of alcohols. The strain was tentatively identified as *P. putida* and designated strain HK5. Most alkane-oxidizing pseudomonads that have been reported grow on 1-octanol (8). However, there are no reports that the ADHs produced from alkane-oxidizing bacteria contain PQQ. This is the first report of a single microorganism that synthesizes three different kinds of quinoprotein ADHs. The present study clearly indicates that the three distinct quinoprotein ADHs are produced at different levels in *P. putida* HK5, depending on the carbon sources in the medium. Although the three ADHs exhibited similar reactivities to primary alcohols, they were distinguished by their induction levels and substrate specificities. ADH I was found in cells grown on

TABLE 7. Kinetic constants of the three ADHs[a]

ADH and substrate	K_m (mM)[b]	V_{max} (U/mg)[b]	V_{max}/K_m
I			
Ethanol	0.163	26.3	161
1-Butanol	1.62	21.5	13.3
1,2-Propanediol	24.9	12.3	0.49
Glycerol	ND[c]	ND	ND
IIB			
Ethanol	5.61	15.4	2.73
1-Butanol	0.105	17.1	163
1,2-Propanediol	10.2	4.27	0.42
Glycerol	ND	ND	ND
IIG			
Ethanol	33.2	11.5	0.34
1-Butanol	0.15	14.7	98.0
1,2-Propanediol	0.226	17.4	77.0
Glycerol	2.40	17.4	5.68

[a] Enzyme activity of ADH I was measured in 50 mM glycine-NaOH buffer (pH 9.0) containing 0.4 mM PMS, 0.22 mM DCIP, 50 mM ethylamine, and various concentrations of substrate. For ADHs IIB and IIG, assays were done in 50 mM Tris-HCl (pH 8.0) containing 20 mM potassium ferricyanide and various concentrations of substrate.

[b] Apparent K_m and V_{max} values were determined from double-reciprocal plots.

[c] ND, not detected.

many kinds of alcohol, but ethanol was the best substrate for production of the enzyme. Monoalcohols bigger than ethanol were effective in producing ADH IIB, and such alcohols were also good substrates for the enzyme. Production of ADH IIG was more restricted in its specificity; only 1,2-propanediol and glycerol were capable of inducing the enzyme. ADH IIB may be important for dissimilation of longer-chain alcohols, because it was induced to a greater extent with these alcohols than was ADH I. Moreover, its kinetic constant showed greater reactivity with 1-butanol than with ethanol. Similarly, ADH IIG may be important for growth on 1,2-propanediol and glycerol.

Further studies will attempt to clarify the induction mechanisms and the physiological functions of the individual ADHs. It is very interesting that *P. putida* HK5 can recognize a small difference between the inducers 1-butanol and 1,2-pro-
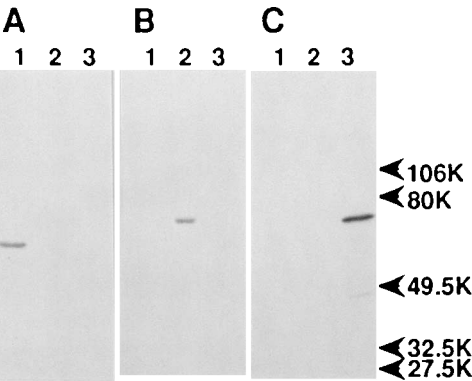

FIG. 5. Immunoblot analysis of the soluble fractions. SDS-PAGE and blotting were done as described for Fig. 4, with soluble fractions (50 μg of protein) of cells grown on the carbon sources indicated above the gel. Antisera used were anti-ADH from *P. aeruginosa* (A), anti-ADH IIB (B), and anti-ADH IIG (C). The positions of the respective enzymes are indicated (arrowheads). Molecular weights are indicated in thousands.

FIG. 4. Immunoblot analysis of ADHs. Purified ADHs (0.5 μg of each protein) were subjected to SDS-PAGE (8% polyacrylamide separation gel) and electroblotted onto a polyvinylidene difluoride membrane. Antisera used were anti-ADH from *P. aeruginosa* (A), anti-ADH IIB (B), and anti-ADH IIG (C). Lanes 1 to 3, ADHs I, IIB, and IIG, respectively. Molecular weights (in thousands) are indicated on the right.
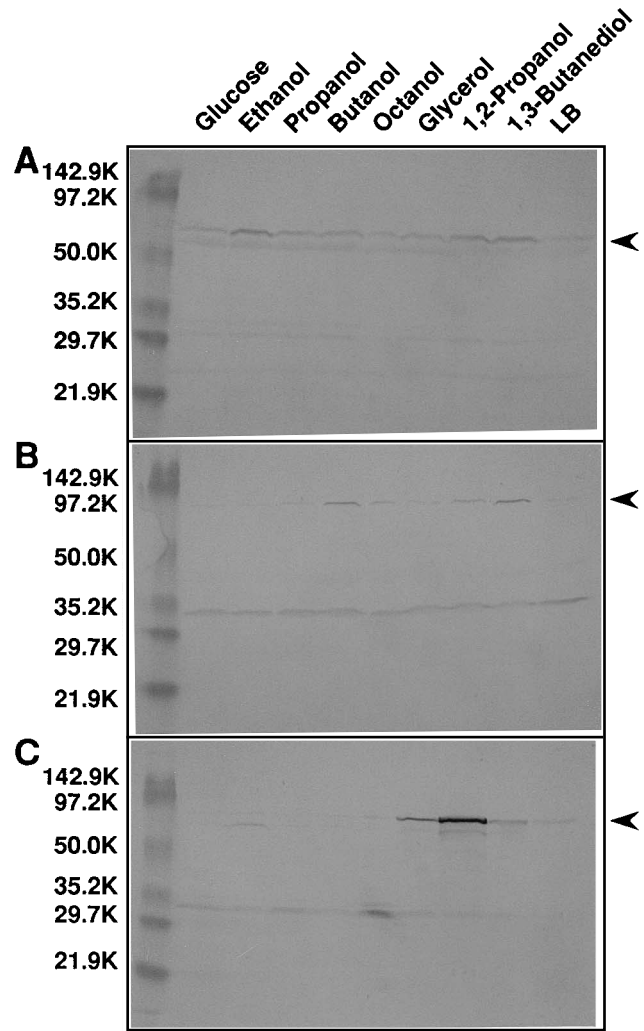
panediol, leading to formation of two different enzymes, ADH IIB and ADH IIG, despite the synthesis of ADHI in many alcohols. It may be possible to utilize the same machinery for heme *c* synthesis in the formation of two quinohemoprotein ADHs. A crucial control mechanism in PQQ biosynthesis might be revealed by comparison of *C. testosteroni* and *P. putida* HK5 to investigate why the former strain cannot produce PQQ, because the two organisms are very similar in many respects. The methanol oxidase system of methylotrophs is known to be composed of soluble methanol dehydrogenase, cytochrome c_H, cytochrome c_L, and membrane-bound cytochrome oxidase (6). On the other hand, the type III ADH which is membrane bound in acetic acid bacteria donates electrons from alcohol to the terminal oxidase via an intramembranous electron acceptor, ubiquinone (17). It is not clear whether the three ADHs from *P. putida* HK5 donate electrons to the electron acceptor in the soluble fraction, like methanol dehydrogenase, or interact directly with the membrane-bound electron transport system. A search for possible electron ac-

ceptors in the soluble fraction and in the membrane fraction will be important for elucidating the alcohol oxidase system in this organism.

When *P. putida* HK5 was grown on D-glucose, a faint ADH I band was occasionally detected in the soluble fraction. Densitometric estimation from activity-stained polyacrylamide gels indicated <2% of the amount of ADH I found in ethanol-grown cells, even when the same amount of protein was loaded and the activity staining was performed in the presence of ethylamine (data not shown). However, immunoblot analysis (Fig. 5) indicated the presence of a protein band cross-reacting with antibody raised against ADH I. Whether the protein band corresponds to ADH I apoenzyme or a similar protein which can cross-react with the antibody should be investigated, because the glucose oxidase system, in which quinoprotein glucose dehydrogenase functions as the primary dehydrogenase, becomes predominant in most aerobic bacteria when D-glucose is supplied as the sole carbon source (17).

Dye-linked ADHs are also known to be involved in the dissimilation of alkanes. At least two such enzymes are induced in cells grown on primary alcohols, α,ω-diols, or alkanes and are thought to be membrane bound (22). Tassin et al. (20) purified a dye-linked membrane-bound ADH which contained no heme *c* from *P. aeruginosa* grown on hydrocarbon. This ADH may be closely related to the type I ADH reported here, but it is not known if the former ADH contains PQQ. Other type I ADHs have included a dye-linked ADH from *Rhodopseudomonas acidophila* 10050 (7) and a PEG dehydrogenase from a *Flavobacterium* sp. (14).

In this study, two different type II ADHs were demonstrated to occur as holoenzymes in the soluble fraction of *P. putida* HK5. The substrate specificity of type II ADH from *C. testosteroni* resembles those of ADHs IIB and IIG, but the former enzyme did not react with secondary alcohols and glycerol (unpublished results). Two other type II ADHs have been reported: an aromatic ADH from *R. acidophila* M402 (23, 24) and ADH from *P. putida* (21). The latter enzyme seems to be closely related to ADH IIB, but, unlike ADH IIB, it was membrane bound and its molecular weight was about 140,000. Hopper et al. (13) have reported that lupanine hydroxylase from a *Pseudomonas* sp. contains both PQQ and heme *c*. Since this enzyme requires an electron acceptor and is thought to act by dehydrogenation of the substrate followed by hydration, it can be included as a type II ADH. For now, it is uncertain whether lupanine hydroxylase is related to ADH IIB or ADH IIG.

A brief comment is necessary to distinguish ADH IIG from an alternative quinoprotein glycerol dehydrogenase produced by *Gluconobacter industrius* (5). The *Gluconobacter* enzyme is localized in the cytoplasmic membrane and does not contain heme *c*. Dihydroxyacetone is the direct reaction product of glycerol oxidation by this enzyme, whereas the reaction product of ADH IIG is presumed to be glyceraldehyde because ADH IIG reacts with 1,3-propanediol but not with 2-propanol. The substrate specificity of glycerol dehydrogenase from *G. industrius* is completely different from that of ADH IIG, which cannot react with various sugar alcohols, such as adnitol, xylitol, and dulcitol. When coupled with a lipase, quinoprotein glycerol dehydrogenase has been used for microdetermination of glycerol in the diagnostic determination of neutral fat. Unlike an NAD-dependent enzyme catalyzing reversible oxidoreduction of glycerol, quinoprotein glycerol dehydrogenase catalyzes glycerol oxidation irreversibly. Thus, quinoprotein glycerol dehydrogenase can determine a trace amount of glycerol released from hydrolysis of neutral fat. For this purpose, ADH IIG should be more useful than the *Gluconobacter* en-

zyme because it is soluble, has a lower apparent K_m for glycerol, and is relatively easy to handle without the presence of any detergents.

The type III ADH is localized in the cytoplasmic membrane of acetic acid bacteria and needs to be solubilized with detergents for purification (3). It consists of three different subunits and contains three hemes *c* in addition to one PQQ, and thus there is much ambiguity about its catalytic mechanism (17). Since the molecular properties of subunit I of the type III ADH resemble those of ADHs IIB and IIG, information obtained for ADH IIB or IIG, including on intramolecular electron flow, may be helpful for understanding and investigation of electron transport in subunit I of the type III ADH, because ADHs IIB and IIG were purified as monomeric dispersed proteins without detergents. Comparison of amino acid sequences of these two quinohemoprotein ADHs and subunit I of type III ADH is also needed.

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