An inducible 1-butanol dehydrogenase, a quinohaemoprotein, is involved in the oxidation of butane by ‘Pseudomonas butanovora’

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INTRODUCTION

‘Pseudomonas butanovora’ is a Gram-negative, rod-shaped bacterium. It was isolated from activated sludge from an oil-refining plant using n-butane as the energy source (Takahashi et al., 1980). Based on morphological, physiological and biochemical characteristics, this organism was grouped in the genus Pseudomonas (Takahashi, 1980). However, the sequence of the 16S rDNA gene is most similar to that of members of the genus Azoarcus (N. Hamamura, personal communication). This genus is characterized by members that degrade aromatic compounds (Krieger et al., 1999; Philipp & Schink, 1998) under anaerobic conditions and other members that are plant epiphytes with the ability to fix nitrogen (Reinhold-Hurek et al., 1993). ‘P. butanovora’ can utilize a variety of organic compounds as growth substrates: C2–C9 n-alkanes, the corresponding primary alcohols, carboxylic acids and some polyvalent alcohols, but not alkenes, sugars or C1 compounds (Takahashi, 1980; Takahashi et al., 1980). Butane-grown ‘P. butanovora’ can degrade several chlorinated aliphatic hydrocarbons (Hamamura et al., 1997), which indicates the potential of butane-grown ‘P. butanovora’ in the bioremediation of sites contaminated with solvents.

Butane-grown ‘Pseudomonas butanovora’ expressed two soluble alcohol dehydrogenases (ADHs), an NAD+-dependent secondary ADH and an NAD+-independent primary ADH. Two additional NAD+-dependent secondary ADHs could be detected when cells were grown on 2-butanol and lactate. The inducible NAD+-independent 1-butanol dehydrogenase (BDH) of butane-grown cells was primarily responsible for 1-butanol oxidation in the butane metabolism pathway. BDH was purified to near homogeneity and identified as a quinohaemoprotein, containing, per mol enzyme, 1 < 0 mol pyrroloquinoline quinone (PQQ) and 0 < 25 mol haem c as prosthetic groups. BDH was synthesized as a monomer of approximately 66 kDa. It has a broad substrate range, including primary alcohols, secondary alcohols, aldehydes, C4 diols and aromatic alcohols. It exhibited the lowest $K_m$ (7 ± 1 μM) and highest $k_{cat}/K_m$ (72 x 10^4 M^-1 s^-1) value towards 1-butanol. BDH exhibited ferricyanide-dependent ADH activity. Calcium ions (up to 10 mM) increased BDH activity substantially. Two BDH internal amino acid sequences showed 73 and 62% identity and 83 and 66% similarity, respectively, when compared with an amino acid sequence of ethanol dehydrogenase from Comamonas testosteroni. The presence of the inducible BDH and secondary ADH may indicate that the terminal and subterminal oxidation pathways are involved in butane degradation of butane-grown ‘P. butanovora’.

Keywords: butane metabolism, 1-butanol dehydrogenase, quinohaemoprotein

Abbreviations: ADH, alcohol dehydrogenase; BDH, 1-butanol dehydrogenase; DCPIP, 2,6-dichlorophenolindophenol; EDH, ethanol dehydrogenase; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate; PQQ, pyrroloquinoline quinone.
The pathways for metabolism of gaseous n-alkanes (C₂–C₆) by some of these organisms have been investigated by several techniques. In all cases, metabolism is initiated by a monoxygenase (Ashraf et al., 1994; Hamamura et al., 1999; Perry, 1980; Stephens & Dalton, 1986; Woods & Murrell, 1989). Terminal oxidation of an alkane leads to the corresponding 1-alcohol, while subterminal oxidation produces the 2-alcohol. Evidence for either, and in certain organisms for both, terminal and subterminal oxidation has accumulated. For example, the terminal oxidation of butane was inferred by the induction of isocitrate lyase in *Mycobacterium vaccae* JOB5 grown on butane or butyrate. Furthermore, isocitrate lyase was not induced when cells were grown on butanone, a predicted intermediate in the subterminal oxidation of butane (Phillips & Perry, 1974). In contrast, subterminal oxidation of propane was suggested by the accumulation of acetone in propane-grown cells (Vestal & Perry, 1969). Moreover, an inducible 2-propanol dehydrogenase was purified from *M. vaccae* JOB5 grown on either propane or 2-propanol (Coleman & Perry, 1985). *Pseudomonas fluorescens* NRRL-B1244 can utilize propane, 1-propanol and 2-propanol as growth substrates. Two soluble NAD⁺-linked alcohol dehydrogenases (ADHs) (one primary ADH and one secondary ADH) were detected in propane-grown cells, which suggested that propane is metabolized through both terminal and subterminal oxidation pathways (Ashraf & Murrell, 1992). The presence of both terminal and subterminal propane oxidation pathways in *Rhodococcus rhodochrous* PNKb1 was suggested because mutants unable to utilize either 1-propanol or 2-propanol were also unable to use propane as a growth substrate, indicating that both alcohols are intermediates of propane metabolism (Ashraf & Murrell, 1992). The terminal oxidation of butane by butane-grown *Nocardia* TB1 was indicated by the accumulation of butyrate in the presence of appropriate inhibitors (Van Ginkel et al., 1987). These results indicate the diversity of butane and propane oxidation pathways, including the diversity of the ADHs involved in alkane metabolism.

The pathway of butane metabolism by butane-grown *P. butanovora* was recently determined to follow the terminal oxidation pathway, that is butane → 1-butanol → butyraldehyde → butyrate (Arp, 1999). Each intermediate (1) accumulated in the presence of appropriate inhibitors, (2) supported cell growth, and (3) stimulated O₂ consumption of butane-grown cells. Although no production of 2-butanol was detected, 2-butanol was consumed and stimulated O₂ consumption by butane-grown *P. butanovora*. Beers (1988) examined the expression of ADH in butane-grown *P. butanovora*. Three soluble ADHs (one primary ADH and two secondary ADHs) that required NAD⁺ as an electron acceptor were found. A membrane-bound, NAD(P)⁺-independent ADH was also described.

In this study, we focused on the characterization of the ADH(s) induced in *P. butanovora* in response to growth on butane. Although butane-grown cells consume 1- and 2-butanol, it was not known if one ADH was oxidizing both substrates. Our goal was to determine the number and specificity of ADHs present in butane-grown *P. butanovora*, and to purify and characterize the inducible ADH primarily involved in the butane metabolism pathway. We report here on the purification and characterization of a quinohaemoprotein, type II ADH induced in butane-grown *P. butanovora*. This report is the first to describe a quinohaemoprotein produced from n-alkane-grown bacteria.

**METHODS**

**Chemicals.** All high-purity alcohols and aldehydes (98–99.99%) were purchased from Sigma and Aldrich. Other chemicals used were analytical grade.

**Bacterial strains and growth conditions.** *P. butanovora* (ATCC 43655) cultures were grown in 12 l bottles at 30 °C with vigorous recirculation of a gas mixture (150 l consisting of 10% v/v, butane; 5% v/v, CO₂; 85%, v/v, air) through the bottle. The growth medium consisted of, per liter, 8 g (NH₄)₂HPO₄, 1.9 g Na₂HPO₄·7H₂O, 2.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.06 g CaCl₂·2H₂O, 0.05 g yeast extract, and 1 ml of the trace element solution described by Wiegent & de Bont (1980). For growth on 1-butanol, 2-butanol or lactate, an appropriate amount of each growth substrate was added to the medium to make the final concentration 4 mM. Butane and CO₂ were omitted from the gas mixture. Cells were harvested at mid- to late-exponential growth phase (~ 45–48 h). Cells were harvested by centrifugation (JA-14; Beckman J2-21) (15 min at 11000 g, 4 °C), resuspended at a ration of 1:1 (w/v) in 25 mM Tris/HCl pH 8.0 and kept at ~ 80 °C until used.

**Preparation of soluble fraction and purification of 1-butanol dehydrogenase (BDH).** All procedures for preparation of the soluble fraction and further purification were performed at 4 °C unless stated otherwise. Unbroken cell suspensions were passed three times through a French pressure cell at 55–62 MPa. Unbroken cells were removed by centrifugation at 11000 g for 15 min. The cell-free extract was centrifuged at 200000 g for 1 h (SW40; Beckman L8-70); the resulting supernatant was the soluble fraction and the sediment was collected as the membrane fraction.

BDH was purified from the soluble fraction prepared from butane-grown cells (see above) by the following steps. In each step, the active fraction(s) were selected primarily by activity stain of non-denaturing gels and then the 1-butanol-dependent phenazine methosulfate (PMS) reductase activity of each active fraction was quantified spectrophotometrically. All active fractions were pooled and dialysed against 25 mM Tris/HCl pH 8.0 overnight before being applied to the next column. (i) Fractionation with ammonium sulfate. To the soluble fraction, ammonium sulfate (to 30% saturation) was slowly added. The resulting precipitate was removed by centrifugation at 14000 g for 30 min. Additional ammonium sulfate was added to the supernatant to reach 70% saturation. The resulting precipitate was collected by centrifugation and redissolved at a ratio of 1:2 (w/v) in 25 mM Tris/HCl buffer pH 8.0. (ii) Q-Sepharose FPLC. The dialysed 30–70% ammonium sulfate fraction was applied to a Q-Sepharose anion exchange FPLC column (Millipore) (2·2 x 10 cm) which had been equilibrated with 25 mM Tris/HCl buffer pH 8.0. The proteins were eluted by a continuous gradient of NaCl (0–0.5 M).
gradient of 0–1 M NaCl in the same buffer. The ADH of interest, which has a pinkish-red colour, eluted at 200–250 mM NaCl, while a secondary ADH eluted at approximately 600 mM NaCl. (iii) 4-Amino-1-butanol affinity chromatography. The dialysed Q-Sepharose fraction containing a primary ADH was applied to 4-amino-1-butanol substrate-analogue affinity column prepared as previously described (Beers, 1988; Lange & Vallee, 1976). The proteins were eluted by a step gradient of 0.05, 0.075, 0.1, 0.2 and 1 M NaCl in 25 mM Tris/HCl pH 8/0. Most proteins did not bind to the column. The ADH of interest eluted at 0.05 M NaCl.

**Enzyme assays.** All enzyme assays were performed at 25 °C. NAD⁺-independent ADH activity was routinely measured as PMS reductase activity. PMS reductase activity was measured spectrophotometrically by monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm in a reaction mixture consisting of 25 mM Tris/HCl pH 8/0, 0.7 mM PMS, 0.1 mM DCPIP, 4 mM NH₄Cl, 0.8 mM KCN and 1 mM 1-butanol in a total volume of 3 ml. The enzyme activity of NAD⁺-dependent ADH was measured by the increase in A₆₅₀ due to NADH in a reaction mixture (total volume 3 ml) consisting of 25 mM Tris/HCl pH 8/0, 2 mM NAD⁺ and the enzyme solution. The endogenous rate of DCPIP or NAD⁺ reduction obtained without substrate was subtracted from the rate observed with substrate. The reaction mixture (2 ml) for ferricyanide reductase activity assay contained 25 mM Tris/HCl pH 8/0, 1 mM potassium ferricyanide, 1 mM 1-butanol and enzyme solution. The enzyme concentration was fixed at 50 nM. One unit of enzyme activity was defined as the amount of enzyme catalysing oxidation of 1 µmol substrate min⁻¹ under the conditions described. Protein content was estimated by the bicinchoninic acid protein assay reagent (Pierce) with bovine serum albumin as a standard (Smith et al., 1986).

**Determination of 1-butanol oxidation and product formation by GC.** The concentration of substrate (alcohol or aldehyde) utilized and type of products (aldehyde or organic acid) formed were determined by GC (Shimadzu GC-8A column equipped with a flame-ionization detector and 60 cm long by 0.1 cm i.d. stainless steel column packed with Porapak Q resin). The column temperature was 150 °C for detection of 1-butanol and butyraldehyde, and 120 °C for detection of 2-butanol and butanone.

**Analytical gel filtration.** The molecular mass of native protein was determined by gel filtration (6 × 30 cm column) with Sephacryl S-300 (Pharmacia). Elution was performed at a flow rate of 0.5 ml min⁻¹ with 25 mM Tris/HCl buffer pH 8/0 containing 0.15 mM NaCl, and was monitored at A₂₈₀. The following molecular mass marker proteins were used for calibration: catalase (M, 240000), bovine serum albumin (M, 66000), carbonic anhydrase (M, 30000) and cytochrome c (M, 13000).

**Electrophoresis.** 10% SDS-PAGE (Mighty Small SE245; Hoefer Scientific Instruments) (Laemmli, 1970; Woods & Murrell, 1989) and Coomassie brilliant blue G250 staining were used to analyse the homogeneity of the purified enzyme.

**Non-denaturing gel and activity staining.** Native protein was applied to non-denaturing gels prepared as above but without SDS. After PAGE, the gel was incubated for 5 min in the dark with a reaction mixture (50 ml) containing 0.01 g nitro blue tetrazolium (NBT), together with 0.7 mM PMS or 1 mM NAD⁺ alone or in combination. Either 1-butanol or 2-butanol was then added to a final concentration of 1 mM and incubated with gentle rocking for another 5 min to develop the colour. The reaction was stopped by rinsing the gel with water.

**Kinetic measurements.** Kinetic parameters were calculated from the initial rates determined with the PMS reductase standard assay described above by varying the concentrations of substrates tested from 2 µM to 1 mM. The enzyme concentration was fixed at 50 nM.

**Preparation of antibodies and immunoblotting.** Purified BDH (2 × 100 μg) well mixed 1:1 (v/v) with TitreMax adjuvant (Sigma) was injected into two New Zealand rabbits. Two weeks later, a booster injection of another 100 μg protein mixed with TitreMax was performed. Another two weeks later, blood was collected, then left at room temperature for 30 min and overnight at 4 °C. After centrifugation at 1000 g for 10 min to remove the erythrocytes, antisera were collected and stored at −80 °C. For immunoblotting analysis, the proteins in the SDS gel after PAGE were transferred electrophoretically with a semi-dry system (Ausubel et al., 1996) onto a PVDF membrane at 10 mA overnight. The membrane was washed with 15 ml Tris-buffered-saline (TBS) (20 mM Tris/HCl, 150 mM NaCl, pH 7.5) before blocking with 15 ml 1% BSA in TBS (TBS with 0.1% Tween 20) for 1 h. The membrane was then incubated with primary antiserum (at a dilution of 1:5000 in TBS) for 1 h. The membrane was washed twice with TBS before incubation with TBS containing secondary antibody [1 mg anti-rabbit IgG (Fc) ml⁻¹; Promega] conjugated with alkaline phosphatase (at a dilution of 1:5000 in TBS) for 30 min. After the membrane had been washed with 20 ml TBS three times, the immunosorbent protein bands were visualized by the addition of 15 ml of Western blue solution (Promega). The colour was developed within 5–20 min. Pre-stained molecular mass marker proteins (Bio-Rad) were included.

**Haem staining.** A lithium dodecylsulfate-PAGE gel was prepared and pre-run overnight (Delepeleire & Chua, 1979). Purified BDH was then applied to the gel and run at 5 mA. Haem staining was performed as described by Thomas et al. (1976).

**Measurement of haem content.** The amount of haem c in the purified BDH was measured from the reduced minus oxidized difference spectra of the pyridine haemochrome (Paul et al., 1953), using a millimolar absorption coefficient of 26.5 (530–534 nm). The oxidized form of pyridine haemochrome was prepared by adding pyridine to 20% (v/v), NaOH to a final concentration of 0.2 M, and potassium ferricyanide to a final concentration of 3 mM, to the enzyme solution. To reduce the reaction mixture, 2 mg sodium dithionite was added to the completely oxidized pyridine haemochrome solution. Cytochrome c from horse heart (Boehringer Mannheim) was used to test the protocol and gave a value of 10 haem per protein molecule.

**Measurement of pyrroloquinoline quinone (PQQ) content.** The PQQ content of purified BDH was determined by HPLC analysis as described by Duine et al. (1983).

**N-terminal and internal amino acid sequence analysis.** To separate the upper and lower bands of the purified BDH, a 13 × 13 cm SDS gel was used. Both upper and lower bands were then blotted onto PVDF membrane and the N-terminal amino acid sequences were determined directly. The internal amino acid sequence was obtained by digesting the purified BDH with endoproteinase Lys-C (Endo Lys-C) in a reaction mixture consisting of 0.2 M Tris pH 9.1, 20% acetonitrile. The enzyme was digested at 37 °C for 20 h, then DTT was added to a final concentration of 5 mM and incubated at 37 °C for another 20 h. Peptides in the digest were separated by HPLC using a Vydac C18 column with a Brownlee guard column. The mobile phase A was 0.1% trifluoroacetic acid.
(TFA) and the mobile phase B was 70% acetonitrile plus 0.1% TFA. Chromatography was performed with a gradient of from 1 to 65% phase B in 75 min. Peaks were monitored at 220 nm and collected for sequence analysis. The prominent fragment was selected for sequencing. The N-terminal and internal amino acid sequencing, including enzyme digestion, were determined by the Biotechnology Laboratory, Institute of Molecular Biology, University of Oregon.

RESULTS

Multiple ADHs in ‘P. butanovora’

Supernatants from cells grown on different substrates were examined for ADH activities using 1-butanol or 2-butanol as substrate. Four distinct activity bands were identified on gels stained for ADH activity when 2-butanol was used as a substrate (Fig. 1g). ADH1, which is the enzyme activity band with the fastest mobility, was detected in the presence of PMS when either 1-butanol (Fig. 1c) or 2-butanol (Fig. 1f) was added. This activity band was not present with either 1- or 2-butanol when NAD⁺ was used without PMS (Fig. 1b, e) and the presence of NAD⁺ together with PMS did not enhance the band intensities (Fig. 1d, g). Therefore, the activity of ADH1 is NAD⁺-independent. The intensity of this band varied with the source of the cells. The intensity of ADH1 activity was highest in butane-grown cells and lowest in lactate-grown cells (Fig. 1c, d; lanes 3 and 4, respectively). The activity of ADH1 was much less when 2-butanol rather than 1-butanol was used as the substrate (Fig. 1f, g). Therefore, this enzyme appears to be a primary ADH and was designated 1-butanol dehydrogenase (BDH).

Three ADHs (ADH2, -3 and -4) were identified as secondary ADHs since they were detected only when 2-butanol was used as a substrate (Fig. 1g). None of these secondary ADHs were detected in 1-butanol-grown cells, while one or two secondary ADHs were detected in 2-butanol-grown, butane-grown and lactate-grown cells.

![Fig. 1](image-url) Non-denaturing gels stained for ADH activity. Native gels after PAGE were stained for 5 min with NBT with NAD⁺ alone (b, e), PMS alone (c, f), or PMS and NAD⁺ together (a, d, g). Gels were then incubated for an additional 5 min with: no substrate (a), 1 mM 1-butanol (b, c, d), or 1 mM 2-butanol (e, f, g). Lanes 1–4 contain cell-free extracts from cells grown in 1-butanol (18 µg protein) (lane 1), 2-butanol (22 µg protein) (lane 2), butane (19 µg protein) (lane 3), and lactate (22 µg protein) (lane 4). Lane 5 contains purified BDH (0.5 µg protein), and lane 6 contains partially purified secondary ADH (20 µg protein) from ‘P. butanovora’. The interface of the stacking gel and the resolving gel are indicated by triangles. Bands corresponding to the four ADHs found in cell-free extracts are indicated.
1-butanol and butane. (a) Western blot analysis of SDS-PAGE of cell-free extracts from: 12 h citrate-grown cells (22 µg protein) (lane 1), 6 h 1-butanol-induced cells (20 µg protein) (lane 2), 12 h 1-butanol-induced cells (20 µg protein) (lane 3), 6 h butane-induced cells (18 µg protein) (lane 4), and 12 h butane-induced cells (23 µg protein) (lane 5). Lane 6 contains purified BDH (2 µg protein). (b) 1-butanol-PMS/DCPIP-dependent activity assay of cell-free extracts from: 12 h citrate-grown cells (1), 6 h 1-butanol-induced cells (2), 12 h 1-butanol-induced cells (3), 6 h butane-induced cells (4) and 12 h butane-induced cells (5).

Our results suggested that an NAD⁺-independent primary ADH (i.e. BDH) was primarily responsible for 1-butanol oxidation. In contrast, it was previously reported that the enzyme responsible for 1-butanol oxidation in butane-grown "P. butanovora" was a membrane-bound NAD(P)⁺-independent primary ADH (Beers, 1988). Therefore, we carefully examined the membrane fraction for ADH activity. Cells grown on 10% butane were harvested, broken, then soluble and membrane fractions were separated as described in Methods. Membrane fractions from different cell growth stages (the beginning of exponential phase, OD₆₀₀ 0–3–0.5; mid- to late-exponential phase, OD₆₀₀ 0–6–0.8; and stationary phase, OD₆₀₀ 1.5–1.7) and solubilized in various concentrations (0.05, 0.1, 0.5 and 1%) of each of several detergents (Triton X-100, Emulgen and dodecyl-β-d-maltoside). None of these preparations showed significant BDH activity (less than

BDH activity is in the soluble fraction

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Table 1. Purification steps and specific activity of BDH from butane-grown ‘P. butanovora’

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)*</th>
<th>Specific activity (U mg⁻¹)*</th>
<th>Purification (-fold)</th>
<th>Recovery (%)</th>
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<td>0.13</td>
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<td>100</td>
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<tr>
<td>30–70% Ammonium sulfate precipitation</td>
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<td>First Q-Sepharose column fractionation</td>
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<td>33</td>
<td>4.8 ± 0.34</td>
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*Activity was determined as 1-butanol-dependent PMS/DCPIP reduction. One unit (U) of activity was defined as the amount of enzyme catalysing the reduction of 1 µmol DCPIP min⁻¹ under the specific conditions of the assay described in Methods.

Fig. 3. 10% SDS-PAGE and Coomassie blue staining of BDH from butane-grown ‘P. butanovora’ after purification steps. All lanes contain approximately 5 µg protein. (a) Lanes: 1 and 10, molecular mass markers; 2, cell-free extract; 3, crude supernatant (after membrane fraction had been removed); 4, after 30–70% ammonium sulfate precipitation; 5, after first Q-Sepharose column fractionation; 6, after first 4-amino-butanol affinity column fractionation; 7, after second Q-Sepharose column fractionation; 8, after second 4-amino-butanol affinity column fractionation; 9, Sephacryl S-300 (for molecular mass verification and subunit determination purposes). (b) The purified BDH was re-run in a large SDS gel to separate two bands (67000 and 65700 Da), which were then blotted onto PVDF membrane and sequenced.

2.5% of the specific activity obtained from soluble fraction (whether NAD⁺ or PMS/DCPIP was used in the activity assay (data not shown). Therefore, the soluble fraction from butane-grown ‘P. butanovora’ was used as an enzyme source for BDH purification.

Purification and physical properties of BDH

BDH activity was the predominant ADH activity in butane-grown cells. The activity was closely correlated with butane metabolism. Therefore, we focused on this enzyme for additional characterization. BDH from butane-grown ‘P. butanovora’ was purified from the soluble fraction to near homogeneity by a six-step procedure (Table 1). Many contaminating proteins were removed by the first anion exchange (Q-Sepharose) column fractionation, which was followed by a butanol affinity column fractionation. Repeating these two column fractionations was sufficient to purify the BDH to near homogeneity. The 37-fold increase in specific activity indicated that BDH accounted for about 2.7% of soluble protein.

A doublet was observed when the purified enzyme was analysed by 10% SDS-PAGE (Fig. 3a). The purified BDH was re-run in a large SDS gel (Fig. 3b) to separate these two bands (67000 and 65700 Da), which were then blotted onto PVDF membrane and sequenced. The N-
An inducible 1-butanol dehydrogenase

Table 2. Alignment of the N-terminal and internal amino acid sequences of BDH from butane-grown ‘P. butanovora’ and the quinohaemoprotein EDH from C. testosteroni

<table>
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<th>Organism</th>
<th>Amino acid sequencea</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
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<tr>
<td>‘P. butanovora’</td>
<td>N-terminal upper band</td>
<td>AGGEWRT-GY-D-A-T</td>
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<tr>
<td></td>
<td>N-terminal lower band</td>
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a Bold letters indicate identical residues.

The secondary ADH observed in butane-grown cells (ADH4) was also partially purified from the soluble fraction by the anion-exchange chromatography step (Fig. 1, lane 6). The partially purified ADH4 did not oxidize 1-butanol (either with NAD+ or PMS), but oxidized 2-butanol when NAD+ was provided.

The molecular mass of BDH, the primary ADH, was estimated to be 66 kDa by SDS-PAGE (Fig. 3) and 69 kDa by gel permeation chromatography (Sephacryl S–300), which indicates that BDH exists as a monomer. This characteristic is similar to other monomeric quinohaemoproteins with a range of molecular masses of 69–73 kDa (Groen et al., 1986; Hopper et al., 1991; Toyama et al., 1995; Zarnt et al., 1997). Because of the sequence similarity to the quinohaemoprotein EDH, the PQQ content of BDH was examined. BDH contains an almost 1:1 molar ratio of PQQ (0.99 nmol (nmol protein)−1), which indicates that PQQ was not lost during the purification. It has been reported that PQQ is non-covalently bound to quinohaemoprotein-type enzymes and can be dissociated by dialysis overnight in Tris/HCl buffer pH 8.0 containing 2 mM EDTA (Zarnt et al., 1997) or by heat treatment (Toyama et al., 1995). BDH exhibits a pinkish-red colour in solution and appears as a red band in non-denaturing gels. A haem-staining band corresponding to a molecular mass of 65 kDa (data not shown) suggested the presence of haem cofactor in this enzyme. BDH as isolated exhibited a typical spectrum of reduced c-type cytochrome with absorption peaks at 416 nm (γ peak), 520 nm (β peak) and 550 nm (α peak) (Fig. 4). When BDH was oxidized...
with 200 μM potassium ferricyanide, the α and β peaks disappeared and the γ peak was shifted to 410 nm (Fig. 4). This spectral change from the reduced form to oxidized form is characteristic of cytochrome c.

The BDH absorption spectrum resembles the absorption spectra obtained for other quinohaemoproteins, for example, the absorption spectra of lupanine hydroxylase, with peaks at 416, 522 and 551 nm (Hopper et al., 1991); of ADH of Pseudomonas putida HK5, with peaks at 418, 523 and 550 nm (Toyama et al., 1995); of tetrahydrofurfuryl ADH fromRalstonia eutropha, with peaks at 419, 523 and 552 nm (Zarnt et al., 1997); and of holoenzyme EDH from C. testosterone (de Jong et al., 1995). When 1 mM 1-butanol was added to the oxidized enzyme, it acted as an electron donor and reduced the c-type haem in BDH (Fig. 4). This result indicated the participation of a cytochrome-c-type prosthetic group in the catalytic activity of BDH. From the ‘reduced minus oxidized’ difference spectrum of pyridine haemochrome (at an absorbance of 534 and 550 nm), a ratio of 1:0 mol haem per 3.8 mol BDH enzyme was calculated. Typically, quinohaemoproteins contain haem at a 1:1 molar ratio (de Jong et al., 1995; Toyama et al., 1995; Zarnt et al., 1997). Although we cannot exclude the possibility, a loss of haem during purification seems unlikely given that c-type haems are generally covalently attached to the protein. Perhaps the substoichiometric haem c content reflects an incomplete assembly of haem c into BDH under the conditions used for growing cells. The presence of two forms of BDH, one with and one without haem c, likely explains the presence of two bands on SDS-PAGE gels (Fig. 3a, b).

**Catalytic properties of BDH**

In addition to the 1-butanol-dependent PMS/DCPIP activity of BDH, we also examined its ferricyanide reductase activity, which is one of the characteristics of quinohaemoproteins. The ferricyanide reductase activity exhibited by BDH from butane-grown ‘P. butanovora’ was 0.62±0.2 μmol min⁻¹ (mg protein)⁻¹ when 1-butanol was used as substrate.

The effect of calcium ions on BDH catalytic activity was determined. The presence of Ca²⁺ ions facilitated the reconstitution of inactive apoenzyme (PQQ-free, but haem-c-containing) quinohaemoprotein from C. testosterone and alkaldoid-degrading Pseudomonas and increased the ADH catalytic activity (de Jong et al., 1995; Groen et al., 1986; Zarnt et al., 1997). Ca²⁺ fulfills a structural role upon reconstitution of apoenzyme with PQQ, facilitating the binding of PQQ to an apoenzyme and activating the bound cofactor (Groen et al., 1986; Zarnt et al., 1997). The activity of BDH from butane-grown ‘P. butanovora’ was significantly increased when Ca²⁺ was added into the reaction mixture. BDH activity with 1-butanol was increased from 3.4±0.2 μmol min⁻¹ (mg protein)⁻¹ (without Ca²⁺) to 4.2±0.01 μmol min⁻¹ (mg protein)⁻¹ with 5 mM CaCl₂ and 4.6±0.2 μmol min⁻¹ (mg protein)⁻¹ with 10 mM CaCl₂. The maximal and saturated activity was reached at a Ca²⁺ concentration of 10 mM. Ca²⁺ was recently reported to have an important role in the catalytic mechanism of quinoprotein-type methanol dehydrogenase (Zheng & Bruice, 1997). The possible roles of Ca²⁺ complexed with PQQ in methanol oxidation include: (i) modest reduction of the pKₐ of the substrate and facilitating the association of substrate to active site, (ii) polarizing the oxygen at the C-5 position of PQQ, and (iii) placing the reaction components in the right positions to react, therefore contributing to the formation of enzyme–substrate complex (Zheng & Bruice, 1997).

BDH activity was increased approximately threefold when ammonium ions (as 4 mM NH₄Cl) were present. A similar result was obtained in tetrahydrofurfuryl ADH from R. eutropha where the activity was increased 2.5-fold in the presence of ammonium sulfate (Zarnt et al., 1997).

The optimum pH for PMS/DCPIP-dependent BDH activity was 8.0. The temperature optimum was 60 °C, at which the specific activity was increased 9.6-fold relative to that at 25 °C. The temperature range of maximum stability of the enzyme was 25–32 °C, within which the loss of BDH activity was insignificant after a 30 min incubation. In contrast, BDH activity was decreased 77% when incubated at 60 °C for the same period of time. BDH activity was stable for more than 6 months when stored in 25 mM Tris/HCl pH 8.0 at −80 °C.

BDH exhibited broad substrate specificity towards various primary alcohols, secondary alcohols, diols, aldehydes and aromatic alcohols (Table 3). Nonetheless, the highest activity was observed with 1-butanol, the butane oxidation product in the terminal oxidation pathway. Therefore, the activities obtained on other substrates were compared to the maximal activity obtained on 1-butanol. Besides 1-butanol, 1-propanol showed a relatively high activity (66%) among the primary alcohols tested. The relative activities among primary alcohols decreased with longer-chain alcohols. Little activity (1–2%) was detected with methanol, which is consistent with the observation that ‘P. butanovora’ cannot grow with either methane or methanol as a substrate (Takahashi, 1980). Among the secondary alcohols tested, BDH exhibited a marked preference towards 2-pentanol and the activity gradually decreased with longer-chain secondary alcohols. DCPIP was slowly reduced by BDH when 2-propanol or 2-butanol was added. In activity-stained native gels, BDH activity bands were also detected when 2-butanol was added (Fig. 1f, g). However, when 2-butanol consumption was determined directly by GC, no 2-butanol consumption was observed. Furthermore, no butanone formation was detected within the 60 min assay (data not shown). The low level of DCPIP reduction activity obtained upon addition of either 2-propanol or 2-butanol was probably due to the presence of contaminants that supported activity. BDH was active towards C₄ diols when one of the hydroxyl groups was
Table 3. Substrate specificity of purified BDH from butane-grown ‘P. butanovora’

Enzyme activity was determined by PMS/DCPIP reduction as described in Methods. Activities are means of at least three replicates. Data are expressed relative to a value of 100% for BDH activity with 1-butanol [48 ± 0.34 μmol min⁻¹ (mg protein)⁻¹].

<table>
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<tr>
<th>Substrate</th>
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<th>Relative activity (%)</th>
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<tr>
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<td>Diols</td>
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<tr>
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<td>2-Nonanol</td>
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<td>Butyraldehyde</td>
<td>78</td>
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at the α-position. Benzyl alcohol and phenol were chosen as the representative aromatic alcohols and were slowly oxidized by BDH. BDH readily oxidized the three aldehydes we tested, with butyraldehyde showing the highest activity. In fact, only 1-butanol was oxidized more rapidly than butyraldehyde.

The kinetic parameters of BDH with 1-butanol and butyraldehyde were determined. At higher concentrations of 1-butanol (> 10 mM), the enzyme activity was decreased. BDH exhibited greater affinity towards 1-butanol (Km 7 ± 1 μM) than towards butyraldehyde (Km 535 ± 13 μM). The term kcat/Km is used to compare the efficiency of catalysis of BDH towards different substrates. 1-Butanol was clearly the preferred substrate with a kcat/Km of 72 × 10⁶ M⁻¹ s⁻¹, which is 51-fold higher than that of butyraldehyde. The product of 1-butanol oxidation was butyraldehyde, as determined by GC. A time course experiment confirmed that BDH prefers 1-butanol to butyraldehyde. 1-Butanol (1 mM) and butyraldehyde (1 mM) were added together as substrates for BDH in a reaction mixture containing PMS/DCPIP in 25 mM Tris/HCl pH 8.0. The oxidation of 1-butanol and butyraldehyde and the formation of their products were determined by GC (Methods). Only 1-butanol was oxidized during the first 60 min, which corresponded with an increase in butyraldehyde. After 1-butanol was completely oxidized, the oxidation of butyraldehyde was initiated and yielded butyric acid. This result is consistent with the 76-fold higher affinity of BDH towards 1-butanol than towards butyraldehyde.

DISCUSSION

In this work, four ADHs were detected in ‘P. butanovora’ grown on different growth substrates. The level of expression of each ADH depended on the carbon source used in the medium. ‘P. butanovora’ grown on butane expressed two soluble ADHs, an NAD⁺-independent primary ADH and an NAD⁺-dependent secondary ADH. The NAD⁺-independent primary ADH has high affinity and activity towards 1-butanol and was expressed at a high level in cells grown on butane or 1-butanol. Therefore, this enzyme (BDH) was considered to be of primary importance in the oxidation of 1-butanol produced from the terminal oxidation of butane. However, our results contradict a report in which a membrane-bound, NAD(P)⁺-independent ADH with an unusually high activity towards methanol (> 60% relative to 1-butanol) was reported to be responsible for the oxidation of butanol in butane-grown ‘P. butanovora’ (Beers, 1988). In our experiments, no 1-butanol oxidation activity could be detected in the membrane fraction.

We purified this soluble, NAD⁺-independent primary ADH from butane-grown ‘P. butanovora’ and showed it to be a quinohaemoprotein. As pointed out by Toyama et al. (1995), there have been no reports of PQQ-containing ADHs associated with alkane oxidation by bacteria. This work is the first report of a quinohaemoprotein induced from alkane-grown bacteria. Interestingly, this inducible BDH from butane-grown ‘P. butanovora’ was found to be different from ADHs induced in other alkane-grown Pseudomonas or other short-, medium- or long-chain-alkane-oxidizing bacteria. ADHs induced in propane-grown R. rhodochrous PNKb1, M. vaccae JOB5 and P. fluorescens NRRL B-1244 were purified and characterized as NAD⁺-dependent secondary ADH(s) (Ashraf & Murrell, 1990, 1992; Coleman & Perry, 1985). Hepante- and octane-grown Pseudomonas aeruginosa strain 473 contain soluble NAD(P)⁺-dependent ADH and at least two NAD(P)⁺-independent quinoprotein (without haem c) primary ADHs (Van Der Linden & Huybrechts, 1969).

In addition to methanol dehydrogenase, other quinoprotein ADHs found in oxidative nonmethylotrophic bacteria have been classified into three groups (types I–III) according to their molecular properties, catalytic properties and localization (Matsushita et al., 1994). The molecular structure of type I ADH resembles that of methanol dehydrogenase, but it has very low affinity for methanol. Type II ADH is a soluble quinohaemoprotein. To date, type II ADHs have been purified from five bacteria: as the apoenzyme, lacking PQQ, from alkald-degrading Pseudomonas (Hopper et al., 1991) and C. testosteroni (de Jong et al., 1995; Groen et al.,
BDH for 1-butanol is much higher than that of quinohaemoprotein group. However, the affinity of osteroni
EDH, indicates that BDH belongs to the type II C
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The physical properties of BDH presented here, in-
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of 1-butanol-dependent PMS
addition of ferricyanide, and back to a reduced spectrum
spectrum that changed to an oxidized spectrum upon
addition of 1-butanol. (iv) A ferricyanide reductase
activity was observed in non-denaturing gels. (ii) Protein corresponding to a
molecular mass of 65 kDa in lithium dodecylsulfa-
PAGE also stained positive for haem. (iii) The
absorption spectrum of purified BDH, with peaks at 416, 520 and 550 nm, was a typical reduced cytochrome c spectrum that changed to an oxidized spectrum upon addition of ferricyanide, and back to a reduced spectrum upon addition of 1-butanol. (iv) A ferricyanide reductase activity was exhibited by BDH; quinoproteins, lacking haem c, such as methanol dehydrogenase, EDH from P. aeruginosa and P. putida, and soluble glucose dehydro-
genase from Escherichia coli, do not react with ferricyanide, suggesting the oxidation of the enzyme by ferricyanide occurs at the haem c site (Dokter et al., 1986; Geerlof et al., 1994; Groen et al., 1984). For a cell extract from 1-butanol-grown P. putida HK5, the ratio of 1-butanol-dependent PMS/DCPIP-reductase activity to ferricyanide-reductase activity was 2:1 (Toyama et al., 1995). BDH from butane-grown ‘P. butanovora’ as isolated expressed, with 1-butanol present, a specific activity ratio of PMS/DCPIP reductase activity to ferricyanide reductase activity of 7:8. This specific activity ratio is consistent with the fact that only one quarter of the BDH as isolated contained haem c.

The physical properties of BDH presented here, in-
cluding high internal sequence similarity with C. test-
osteroni EDH, indicates that BDH belongs to the type II quinohaemoprotein group. However, the affinity of BDH for 1-butanol is much higher than that of C. testosteroni EDH for ethanol or of R. eutropha tetra-
hydrofurfuryl ADH for n-pentanol (Geerlof et al., 1994; Zarnit et al., 1997). The $K_m$ of BDH towards 1-butanol ($7 \pm 1 \mu M$) is the lowest $K_m$ reported among other quinohaemoprotein ADHs obtained from ethanol-grown C. testosteroni ($K_m$ 2230 ± 64 μM towards ethanol; Geerlof et al., 1994) or 1-butanol grown P. putida HK5 ($K_m$ 105 μM towards 1-butanol; Toyama et al., 1995). Coupled with much lower affinity for butyraldehyde ($K_m$ 535 ± 13 μM), 1-butanol is clearly preferred over butyraldehyde by BDH. In contrast, C. testosteroni EDH has a higher affinity for the aldehyde ($K_m$ for acetaldehyde 669 ± 22 μM), such that acetic acid is produced even in the presence of ethanol.

It was recently demonstrated that butane metabolism by ‘P. butanovora’ was mainly via a terminal oxidation pathway (Arp, 1999). Up to 90% of the butane consumed was accounted for as 1-butanol. The results of this work are consistent with terminal oxidation. The predominant ADH activity in butane-grown cells is much more efficient towards 1-butanol than 2-butanol. Furthermore, the enzyme is expressed at the highest level observed in butane-grown cells. However, butane-grown cells also expressed a low level of 2-butanol-dependent ADH activity that was not present in 1-butanol-grown cells. This result may reflect a low level of subterminal butane oxidation involved in butane metabolism in ‘P. butanovora’. Studies of the genes encoding each of these enzymes, including expression experiments and gene disruptions, will be pursued to further characterize the role of each ADH in butane metabolism.

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monas putida (pWWO) growing on succinate in continuous


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