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

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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 ($7 \pm 1 \mu\text{M}$) and highest k_{cat}/K_m ($72 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) 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

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.

Except for the Gram-negative bacteria '*P. butanovora*' and *Pseudomonas* sp. strain CRL71 (Hou *et al.*, 1983), most butane-oxidizing bacteria are members of the Gram-positive *Corynebacterium*–*Nocardia*–*Mycobacterium*–*Rhodococcus* complex (Ashraf *et al.*,

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.

1994). The pathways for metabolism of gaseous *n*-alkanes (C_2 – C_4) by some of these organisms have been investigated by several techniques. In all cases, metabolism is initiated by a monooxygenase (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 $\rightarrow$ 1-butanol $\rightarrow$ butyraldehyde $\rightarrow$ butyrate (Arp, 1999). Each intermediate (1) accumulated in the presence of appropriate inhibitors, (2) supported cell growth, and (3) stimulated O_2 consumption of butane-grown cells. Although no production of 2-butanol was detected, 2-butanol was consumed and stimulated O_2 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 can

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, CO_2 ; 85%, v/v, air) through the bottle. The growth medium consisted of, per liter, 8.0 g $(NH_4)_2HPO_4$, 1.9 g $Na_2HPO_4 \cdot 7H_2O$, 2.0 g KH_2PO_4 , 0.5 g $MgSO_4 \cdot 7H_2O$, 0.06 g $CaCl_2 \cdot 2H_2O$, 0.05 g yeast extract, and 1 ml of the trace element solution described by Wiegant & 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 CO_2 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 ratio 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 buffer pH 8.0 at 4 °C overnight before being applied to the next column. (i) Fractionation with ammonium sulfate. To the soluble fraction, ammonium sulfate (to 30% saturation) was gradually 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 $\times$ 10 cm) which had been equilibrated with 25 mM Tris/HCl buffer pH 8.0. The proteins were eluted by a continuous

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 A₃₄₀ 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 (1.6 × 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_r 240 000), bovine serum albumin (M_r 66 000), carbonic anhydrase (M_r 30 000) and cytochrome *c* (M_r 13 000).

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 TBST (TBS with 0.1% Tween 20) for 1 h. The membrane was then incubated with primary antiserum (at a dilution of 1:5000 in TBST) for 1 h. The membrane was washed twice with TBST before incubation with TBST containing secondary antibody [1 mg anti-rabbit IgG (Fc) ml⁻¹; Promega] conjugated with alkaline phosphatase (at a dilution of 1:5000 in TBST) for 30 min. After the membrane had been washed with 20 ml TBST 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 (Delepelaire & 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 *c* 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 (550–534 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 1.0 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 to two secondary ADHs were detected in 2-butanol-grown, butane-grown and lactate-grown cells

Fig. 1. 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.

Fig. 2. Induction of BDH during growth of '*P. butanovora*' on 1-butanol and butane. (a) Western blot analysis of SDS-PAGE of cell-free extracts of '*P. butanovora*' grown in 5 mM citrate and induced for BDH activity by 1-butanol or butane for 6 and 12 h. The antibodies used were raised against purified BDH from butane-grown '*P. butanovora*'. Lanes 1–5 contain 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).

(Fig. 1g). All three cell extracts contained ADH4, which has the slowest mobility in the non-denaturing gel. 2-Butanol-grown and lactate-grown cells contained additional secondary ADHs, designated ADH2 and ADH3, respectively. Butane-grown cells exhibited little ADH2 activity and no ADH3 activity. These secondary ADHs (ADH2, -3 and -4) all required NAD⁺ for activity and were therefore considered NAD⁺-dependent. Although no activity was observed with PMS alone, the NAD⁺-dependent activity also required the presence of PMS (Fig. 1e, f, g). The failure to observe activity with NAD⁺ alone most likely indicated that NAD⁺ cannot act as sole electron acceptor under the conditions tested and indicated a role for PMS in mediating electron transfer from NADH to NBT (Hisada & Yagi, 1977; Nakamura *et al.*, 1982). The slowly migrating bands that appeared in both the presence and absence of butanol were not attributable to BDH activity and likely came from nonspecific interactions of the NBT with proteins on the gels (Van Noorden *et al.*, 1985).

BDH is induced during growth of '*P. butanovora*' on butane

Growth-substrate-dependent differences in the induction of primary and secondary ADH activities in '*P.*

butanovora' were apparent from the activity-stained non-denaturing gels (Fig. 1c, d). To quantify these differences, BDH activity was determined by spectrophotometric assay in extracts of cells grown on butane, 1-butanol, 2-butanol or lactate. The highest activity was present in cells grown on butane [32.9 ± 1.7 nmol min⁻¹ (mg protein)⁻¹]. The activities of extracts of cells grown on 1-butanol, 2-butanol or lactate were 10.6 ± 0.5 , 4.4 ± 0.1 and 1.9 ± 0.1 nmol min⁻¹ (mg protein)⁻¹, respectively. The activities determined in solution correlated with the intensities of BDH activity bands on native gels.

To further confirm the correlation of BDH activity with butane metabolism, the induction of BDH upon exposure of cells to butane was examined. Cells were grown in citrate (5 mM) because this substrate does not repress the induction of butane metabolism when butane is provided. In contrast, lactate and succinate induce catabolic repression in *Pseudomonas* (Duetz *et al.*, 1994; Yuste *et al.*, 1998). When cells reached an OD₆₀₀ of 0.6 (2 d), they were divided into three parts. One part remained in the citrate-containing medium, another part received 10% butane in the gas phase, and the third part was supplemented with 1-butanol (to 4 mM). Cells were incubated at 30 °C with shaking. After 6 and 12 h, samples of cells were harvested, washed and broken. Further investigation of the induction of BDH activity was performed by Western blot analysis. The results from the activity assay were consistent with the increases of cross-reacting band intensities in Western blot analysis. As shown in Fig. 2(a), the cross-reacting band of an inducible BDH was first seen after cells were incubated with 1-butanol for 6 h. After 12 h, clearly visible cross-reacting bands were present in cell-free extracts of 1-butanol-induced and butane-induced cells, but not in citrate-grown cells. The 1-butanol-PMS/DCPIP-dependent activity assay (Fig. 2b) showed 22-fold and 30-fold increases in BDH activity after citrate-grown cells were induced by 1-butanol and butane for 12 h, respectively.

BDH activity is in the soluble fraction

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

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

Purification step	Total protein (mg)	Total activity (U)*	Specific activity (U mg ⁻¹)*	Purification (-fold)	Recovery (%)
Crude supernatant	1921	250	0.13	1	100
30–70% Ammonium sulfate precipitation	840	308	0.37 ± 0.01	3	123
First Q-Sepharose column fractionation	134	208	1.6 ± 0.06	12	83
First 4-amino-butanol affinity column fractionation	43	143	3.3 ± 0.02	26	57
Second Q-Sepharose column fractionation	16	55	3.5 ± 0.04	27	22
Second 4-amino-butanol affinity column fractionation	7	33	4.8 ± 0.34	37	13

* 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 (67 000 and 65 700 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 (67 000 and 65 700 Da), which were then blotted onto PVDF membrane and sequenced. The N-

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*

Organism		Amino acid sequence*	Identity (%)	Similarity (%)
' <i>P. butanovora</i> '	N-terminal upper band	AGGEWRT-GYD-A-T	73	83
	N-terminal lower band	AGGEWRTAGY-DA-T		
' <i>P. butanovora</i> '	Internal sequence	... K PMS YAPQTGLAYFPAQNIP ...	62	66
<i>C. testosteroni</i>	Internal sequence (427–446)	... H PMS FNPTGLVYLPQNVP ...		
' <i>P. butanovora</i> '	Internal sequence	... K G G GIPNLGYSTAETIAHL DQFVFK ...	62	66
<i>C. testosteroni</i>	Internal sequence (646–670)	... R G G NIPNLGYMDASYIENLP FVFK ...		

* Bold letters indicate identical residues.

terminal amino acid sequence obtained from each band of BDH revealed that, in fact, they belonged to the same enzyme (Table 2). This N-terminal amino acid sequence revealed no strong similarity to any N-terminal amino acid sequence in the databases. This result was not surprising since it has been previously reported that there is generally a low similarity in the N-terminal part of this type of protein (Stoorvogel *et al.*, 1996; Zarnt *et al.*, 1997). To obtain internal amino acid sequences from BDH, an endoproteinase Lys-C digestion was performed and two fragments were purified by HPLC and sequenced. Two alignments of the internal amino acid sequences showed high similarities to parts of quinohaemoprotein ethanol dehydrogenase (EDH) from ethanol-grown *Comamonas testosteroni*, one having an identity of 73 % and a similarity of 83 % to a part of the EDH amino acid sequence (residues 427–446) and the other one having an identity of 62 % and a similarity of 66 % to a part of the EDH amino acid sequence (residues 646–670) (Table 2).

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

Fig. 4. UV/visible spectra of purified BDH (10 μM) from butane-grown '*P. butanovora*' measured in 25 mM Tris/HCl pH 8.0. —, Purified BDH; ---, BDH oxidized with 200 μM potassium ferricyanide; —·—, BDH re-reduced by the addition of 1 mM 1-butanol to the oxidized enzyme.

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 from *Ralstonia eutropha*, with peaks at 419, 523 and 552 nm (Zarnt *et al.*, 1997); and of holoenzyme EDH from *C. testosteroni* (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 $0.62 \pm 0.2 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ when 1-butanol was used as substrate.

The effect of calcium ions on BDH catalytic activity was determined. The presence of Ca^{2+} ions facilitated the reconstitution of inactive apoenzyme (PQQ-free, but haem-*c*-containing) quinohaemoprotein from *C. testosteroni* and alkaloid-degrading *Pseudomonas* and increased the ADH catalytic activity (de Jong *et al.*, 1995; Groen *et al.*, 1986; Zarnt *et al.*, 1997). Ca^{2+} fulfils 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 Ca^{2+} was added into the reaction mixture. BDH activity with 1-butanol was increased from $3.4 \pm 0.2 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (without Ca^{2+}) to $4.2 \pm 0.01 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ with 5 mM CaCl_2 and $4.6 \pm 0.2 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ with 10 mM CaCl_2 . The maximal

and saturated activity was reached at a Ca^{2+} concentration of 10 mM. Ca^{2+} was recently reported to have an important role in the catalytic mechanism of quinoprotein-type methanol dehydrogenase (Zheng & Bruce, 1997). The possible roles of Ca^{2+} complexed with PQQ in methanol oxidation include: (i) modest reduction of the pK_a 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 & Bruce, 1997).

BDH activity was increased approximately threefold when ammonium ions (as 4 mM NH_4Cl) 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 C_4 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 [$4.8 \pm 0.34 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$].

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Primary alcohols		Diols	
Methanol	2	1,2-Propanediol	4
Ethanol	34	1,3-Propanediol	5
1-Propanol	66	1,2-Butanediol	30
1-Butanol	100	1,3-Butanediol	33
1-Pentanol	45	1,4-Butanediol	24
1-Hexanol	29	2,3-Butanediol	2
1-Heptanol	31	1,4-Pentanediol	1
1-Octanol	19	2,4-Pentanediol	2
1-Nonanol	9		
Secondary alcohols		Aromatic alcohols	
2-Propanol	5	Benzyl alcohol	11
2-Butanol	9	Phenol	13
2-Pentanol	68		
2-Hexanol	56	Aldehydes	
2-Heptanol	49	Acetaldehyde	54
2-Octanol	42	Propionaldehyde	61
2-Nonanol	35	Butyraldehyde	78

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 ($> 10 \text{ mM}$), the enzyme activity was decreased. BDH exhibited greater affinity towards 1-butanol ($K_m 7 \pm 1 \mu\text{M}$) than towards butyraldehyde ($K_m 535 \pm 13 \mu\text{M}$). The term k_{cat}/K_m is used to compare the efficiency of catalysis of BDH towards different substrates. 1-Butanol was clearly the preferred substrate with a k_{cat}/K_m of $72 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, 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). Heptane- 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 & Huybregtse, 1969).

In addition to methanol dehydrogenase, other quinoprotein ADHs found in oxidative nonmethylophilic 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 alkaloid-degrading *Pseudomonas* (Hopper *et al.*, 1991) and *C. testosteroni* (de Jong *et al.*, 1995; Groen *et al.*,

1986); and as the active holoenzyme from *P. putida* HK5 (Toyama *et al.*, 1995), *Rhodopseudomonas acidophila* M402 (Yamanaka & Tsuyuki, 1983) and *R. eutropha* (Zarnt *et al.*, 1997). Type III ADH is a membrane-bound ADH found in the cytoplasmic membrane of acetic acid bacteria.

The characterization of purified BDH from butane-grown '*P. butanovora*' indicates that this enzyme is a type II NAD⁺-independent ADH or quinohaemoprotein (Goodwin & Anthony, 1998). This is also the first report of a type II NAD⁺-independent ADH synthesized by an *Azoarcus*-like bacterium. PQQ, a prosthetic group in quinohaemoprotein, is expected to participate directly in the oxidation of alcohols and aldehydes (Groen *et al.*, 1986). Apo-EDH, lacking PQQ but containing haem *c*, has no activity with alcohol or aldehyde substrates. The presence of haem *c* as a functional prosthetic group in BDH was indicated by the following results. (i) A red band, which also stained for ADH activity, was observed in non-denaturing gels. (ii) Protein corresponding to a molecular mass of 65 kDa in lithium dodecylsulfate-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 dehydrogenase 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, including high internal sequence similarity with *C. testosteroni* 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* tetrahydrofurfuryl ADH for *n*-pentanol (Geerlof *et al.*, 1994; Zarnt *et al.*, 1997). The K_m of BDH towards 1-butanol ($7 \pm 1 \mu\text{M}$) is the lowest K_m reported among other quinohaemoprotein ADHs obtained from ethanol-grown *C. testosteroni* (K_m $2230 \pm 64 \mu\text{M}$ towards ethanol; Geerlof *et al.*, 1994) or 1-butanol grown *P. putida* HK5 (K_m $105 \mu\text{M}$ towards 1-butanol; Toyama *et al.*, 1995). Coupled with much lower affinity for butyraldehyde (K_m $535 \pm 13 \mu\text{M}$), 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 $669 \pm 22 \mu\text{M}$), 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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