Long-chain alcohol and aldehyde dehydrogenase activities in *Acinetobacter calcoaceticus* strain HO1-N

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(Received 4 February 1992; accepted 28 May 1992)

Three alcohol dehydrogenases have been identified in *Acinetobacter calcoaceticus* sp. strain HO1-N: an NAD⁺-dependent enzyme and two NADP⁺-dependent enzymes. One of the NADP⁺-dependent alcohol dehydrogenases was partially purified and was specific for long-chain substrates. With tetradecanol as substrate an apparent K_m value of 5.2 μ M was calculated. This enzyme has a pI of 4.5 and a molecular mass of 144 kDa. All three alcohol dehydrogenases were constitutively expressed. Three aldehyde dehydrogenases were also identified: an NAD⁺-dependent enzyme, an NADP⁺-dependent enzyme and one which was nucleotide independent. The NAD⁺-dependent enzyme represented only 2% of the total activity and was not studied further. The NADP⁺-dependent enzyme was strongly induced by growth of cells on alkanes and was associated with hydrocarbon vesicles. With tetradecanal as substrate an apparent K_m value of 0.2 μ M was calculated. The nucleotide-independent aldehyde dehydrogenase could use either Wüster's Blue or phenazine methosulphate (PMS) as an artificial electron acceptor. This enzyme represents approximately 80% of the total long-chain aldehyde oxidizing activity within the cell when the enzymes were induced by growing the cells on hexadecane. It is particulate but can be solubilized using Triton X-100. The enzyme has an apparent K_m of 0.36 mM for decanal.

Introduction

Acinetobacter calcoaceticus strain HO1-N is well known as an alkane-utilizing organism capable of growing on long-chain alkanes and their derivatives. Alkane metabolism is thought to follow the accepted pathway for monoterminal oxidation in bacteria (Makula & Finnerty, 1968) – route (1) –

although hydroperoxides have also been proposed in an alternative route (2)

not involving alcohols (Finnerty 1977, 1988). The organism readily oxidizes n-alkyl hydroperoxides (Finnerty *et al.*, 1962). The present work has been concerned with long-chain alcohol and aldehyde dehydrogenases which may be involved in the mono-

terminal oxidation of n-alkanes and their conversion to the corresponding fatty acids.

Whilst some bacteria such as *Pseudomonas aeruginosa* (van der Linden & Huybregste, 1969; Tassin *et al.*, 1973), *Pseudomonas putida* (Benson & Shapiro, 1976) and *A. calcoaceticus* (Taichert *et al.*, 1975; Jirausch *et al.*, 1986) possess nucleotide-independent alcohol dehydrogenases, no nucleotide-independent aldehyde dehydrogenase has previously been found to be involved in long-chain alkane oxidation (see Singer & Finnerty, 1985a; Kleber *et al.*, 1983). The nucleotide-dependent aldehyde dehydrogenases have been reviewed by Sorger & Aurich (1978). Our work presents evidence which shows that *Acinetobacter* sp. strain HO1-N possesses an inducible, long-chain aldehyde dehydrogenase which is nucleotide independent and which can utilize Wüster's Blue or phenazine methosulphate (PMS) as artificial electron acceptor. The high activity of this previously unknown enzyme suggests that the pathway for monoterminal oxidation of alkanes may need further study.

Methods

Culture conditions and extract preparation. *Acinetobacter calcoaceticus* strain HO1-N (ATCC 14987) was grown on minimal salts media

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Abbreviations: DCP, dichlorophenolindophenol; PMS, phenazine methosulphate; TMPD, tetramethyl-*p*-phenylenediamine hydrochloride.

(Jayasuriya, 1955) with 0.1% (w/v) yeast extract and one of the following as carbon source: 0.5% (v/v) *n*-hexadecane, 0.5% (w/v) *n*-hexadecanol, 1.0% (w/v) sodium succinate, 1.0% (w/v) sodium acetate. Stock cultures were maintained on nutrient agar slopes and subcultured into 250 ml shake flasks containing 100 ml of nutrient broth. After 8 h growth, an 8 ml sample of this culture was transferred to a 1 litre vortex-aerated bottle containing 800 ml of the above medium. Cultures were harvested during the late exponential growth phase. Cell-free extract was produced by passing the bacteria through a French pressure cell at 83 MPa three times. Approximately 5 mg DNAase I was added before the second passage. The extract was centrifuged at 5000 *g* for 30 min at 4 °C and the supernatant fraction was taken as the cell-free extract.

Protein assays. Protein assays were carried out using the Bradford (1976) method with bovine serum albumin as a standard. Detergent-solubilized proteins were assayed using bicinchoninic acid (BCA) protein assay reagent (Smith *et al.*, 1985).

Enzyme assays. Nucleotide-dependent enzymes. The NAD(P)⁺-dependent dehydrogenase enzymes were assayed fluorometrically in a filter instrument of the type described by Dalziel (1962). The assay contained 3.78 ml 20 mM-glycine/NaOH buffer, pH 9.5, 20 µl of enzyme preparation, 100 µl 10 mM-NAD(P)⁺ and 20 µl 10 mM-substrate dissolved in peroxide-free dioxan. The activity was measured at 25 °C by following the increase in fluorescence as NAD(P)H was formed.

Nucleotide-independent enzymes. Alcohol and aldehyde dehydrogenase activities were determined by measuring the rate of 2,6-dichlorophenolindophenol (DCPIP) reduction at 25 °C and 600 nm. The assay system contained 2.58 ml 20 mM-sodium phosphate buffer, pH 7.5, 100 µl PMS (5 mg ml⁻¹), 200 µl DCPIP (3 mM), 20 µl of enzyme solution and 100 µl 10 mM-substrate dissolved in peroxide-free dioxan.

The Würster's-Blue-dependent activity was assayed spectrophotometrically at 600 nm. The assay system contained 2.79 ml 20 mM-sodium phosphate buffer, pH 7.5, 10 µl of enzyme preparation, 100 µl Würster's Blue (0.6 mg ml⁻¹) (made up fresh daily) and 100 µl 10 mM-substrate dissolved in dioxan.

In general, assays were reproducible to within $\pm 5\%$. In the kinetic studies assays were done in triplicate.

Preparation of Würster's Blue. This method is based on that of Michaelis & Granick (1943), with minor alterations: tetramethyl-*p*-phenylenediamine hydrochloride (TMPD) (4 g) was dissolved in a solution of 70 ml water and 115 ml methanol. Sodium perchlorate (50 g) was dissolved in this mixture followed by 127 ml aqueous bromine solution (50% saturated), added dropwise. The mixture was then cooled to -10 °C, filtered through Whatman no. 1 paper and washed several times with small portions of ice-cold methanol and then abundantly with dry diethyl ether. The dry, brown, metallic crystals were stored in a desiccator over P₂O₅ in the dark.

An operational extinction coefficient was determined for each batch of the dye. A known quantity of Würster's Blue was dissolved in water (at a concentration of about 0.6 mg ml⁻¹) and 100 µl of this was added to a cuvette containing 3.9 ml of water. The absorbance was measured at 600 nm against water and the mixture was then completely reduced by adding a few grains of sodium dithionite. The molecular mass of Würster's Blue was taken as 263 Da and the change in absorbance at 600 nm on the addition of dithionite was used to calculate the exact extinction coefficient (values of ϵ of approximately 10000 M⁻¹ cm⁻¹ were usually obtained).

Partial purification of NADP⁺-alcohol dehydrogenase. The crude cell-free extract (about 15 ml, 6 mg ml⁻¹) was applied to a Sephacryl superfine S-300 column with a packed volume of 650 cm³ previously equilibrated with 50 mM-HEPES/NaOH buffer, pH 7.5. The column was run at 4 °C with the equilibration buffer, at a flow rate of about

3 ml min⁻¹. The enzyme was eluted between 300 to 380 ml. The fractions with the highest specific activity were pooled and then applied to a Reactive Red A agarose column previously equilibrated with 50 mM-HEPES/NaOH buffer, pH 7.5. The enzyme was selectively eluted from the column using 15 mM-NADP⁺ in 200 mM-HEPES/NaOH buffer, pH 7.5. The eluate was dialysed overnight against 20 mM-HEPES/NaOH buffer, pH 7.5, to remove residual NADP⁺. The dialysis residue was then subjected to pressure filtration and the concentrated enzyme preparation could then be stored without loss of activity under liquid nitrogen.

Partial purification of NADP⁺-aldehyde dehydrogenase. The initial purification step is as for the alcohol dehydrogenase. Following application of the cell-free extract to the Sephacryl S-300 column the aldehyde dehydrogenase was found to be present in the cloudy fractions (200 to 300 ml) of the eluate. The fractions with the highest specific activity were pooled. Acetone (90%, v/v) cooled to -20 °C was added dropwise with constant stirring to the pooled enzyme preparation in a salt/ice bath, to give a final concentration of 30% (v/v). The extract was immediately centrifuged at 4 °C for 20 min, 48000 *g*. The pellet was resuspended in 20 mM-sodium phosphate buffer, pH 7.5, with 1 mM-dithiothreitol and 1 mM-EDTA. The resuspended pellet was again centrifuged to give a supernatant and pellet. The supernatant fraction was dialysed overnight against 20 mM-sodium phosphate buffer, pH 7.5, 1 mM-DTT to remove residual acetone. The dialysis residue contained the partially purified enzyme.

Preparation of solubilized aldehyde dehydrogenase. The crude cell-free extract was centrifuged at 4 °C, 200000 *g* for 1.5 h. The pellet was resuspended in 50 mM-HEPES/NaOH buffer, pH 7.5, containing 5% (w/v) sucrose and centrifuged as before. The pellet from this centrifugation was resuspended to a protein concentration of 4 to 5 mg ml⁻¹. Triton X-100 (1%, v/v) was added and the extract was stirred for 20 min. The preparation was then centrifuged again as before and the supernatant contained the solubilized Würster's-Blue-dependent aldehyde dehydrogenase.

Gas chromatography of alkanes and fatty acids. Fractions from the Sephacryl S-300 column (5 ml) containing the aldehyde dehydrogenase were adjusted to pH 1 with conc. H₂SO₄ and extracted three times with an equal volume of diethyl ether. The combined ether fractions were evaporated under nitrogen then dried under vacuum at 50 °C over P₂O₅. The residue was then methylated using trimethylsulphonium hydroxide (Butte, 1983) and analysed by gas chromatography.

A Phillips PU 4500 gas chromatograph, fitted with a flame ionization detector and integrator, was used to analyse the samples. The 10% (w/v) diethylene glycol succinate column was set at 160 °C with the injector and detector ports set at 180 °C. The sample constituents were identified by running appropriate standards of alkanes, alcohols and fatty acid methyl esters. Each sample was re-run using a column temperature of 193 °C with the ports at 210 °C.

Molecular mass determination. Sephacryl superfine S-300 was equilibrated with 50 mM-HEPES buffer, pH 7.5, and loaded into a 1.4 cm diam. column. The column was attached to a LKB FPLC system and run at a flow rate of 0.8 ml min. The packed height of the column was 89.5 cm. The column was calibrated with cytochrome *c* (12400 Da), carbonic anhydrase (29000 Da), bovine serum albumin (66000 Da), yeast alcohol dehydrogenase (150000 Da) and β -amylase (200000 Da).

Polyacrylamide gel electrophoresis. A non-denaturing gel was run according to the method of Hames (1981). A discontinuous buffer system was used in a mini-gel apparatus and the concentration of acrylamide in the resolving gel was 10% (w/v). The sample of cell-free extract was diluted 50% in the sample preparation mixture and 15 µl was applied to each sample well. The gel was run at 4 °C, 80 V (constant

Table 1. Total activities and specific activities of alcohol and aldehyde dehydrogenases in *Acinetobacter calcoaceticus* HO1-N grown on various carbon sources

The enzymes were assayed in the crude cell-free extract according to the conditions stated in Methods using, respectively decanol and decanal as substrates. Assays were done in duplicate and were reproducible to within $\pm 5\%$; mean values are recorded. TA, total activity ($\mu\text{mol min}^{-1}$ in 3 g wet wt cells); SA, specific activity [nmol min^{-1} ($\text{mg protein})^{-1}$].

Growth substrate	Enzymes and cofactors:									
	Alcohol dehydrogenase				Aldehyde dehydrogenase					
	NAD ⁺		NADP ⁺		NAD ⁺		NADP ⁺		Würster's Blue	
	TA	SA	TA	SA	TA	SA	TA	SA	TA	SA
Hexadecane	2.3	26	22	250	3.4	39	20	230	170	1940
Hexadecanol	3.9	35	22	200	3.9	36	26	240	140	1300
Sodium succinate	1.8	10.5	22	130	2.7	17	1.0	6	40	380
Sodium acetate	2.0	20	21	210	2.8	28	1.7	17	30	740

voltage) until the front was 1 cm from the base of the gel. The gel was cut and stained for activity. The activity stain contained 40 ml 20 mM-sodium phosphate buffer, pH 8.0, 2 mg PMS, 8 mg Nitroblue tetrazolium and 2 ml 10 mM-substrate in dioxan. The nucleotide-dependent dehydrogenases required the presence of NAD(P)⁺ (10 ml, 10 mM). The gel was soaked in the activity stain for 30 min at 30 °C in the dark, after which time the activity bands were visible. The gel could later be stained for protein using the one-step Coomassie Blue stain (Zehr *et al.*, 1989).

Materials. Yeast extract, nutrient agar and nutrient broth were from Oxoid. Hexadecane, alcohols, aldehydes, Nitroblue tetrazolium, PMS, TMPD, DCPIP, DNAase, bovine serum albumin, Reactive Red A agarose, Sephacryl S-300 and the molecular mass markers for gel filtration were from Sigma. BCA protein reagent, Triton X-100 (10%, v/v, peroxide-free) and Brij-35 (10%, v/v, peroxide-free) were from Pierce and Warriner, Chester, UK. NAD⁺ was from Park Scientific, Northampton, UK. Dithiothreitol was from Boehringer Mannheim.

Results

Acinetobacter calcoaceticus strain HO1-N was grown on a variety of carbon sources and the crude cell-free extract from each batch of cells was assayed for long-chain alcohol and aldehyde dehydrogenase activity according to the conditions in Methods. NAD⁺- and NADP⁺-dependent alcohol and aldehyde dehydrogenase activities were found as expected (Table 1). In addition, a third and very active aldehyde dehydrogenase was found. This enzyme was nucleotide independent and was capable of utilizing the artificial electron acceptors Würster's Blue and PMS.

Apart from the NADP⁺-dependent aldehyde dehydrogenase the total activities (Table 1) of the NAD⁺- and NADP⁺-dependent enzymes do not appear to depend on the choice of carbon source. The NAD⁺-dependent alcohol dehydrogenase activity is low and represents only

about 10% of the total alcohol oxidizing activity of the cell. It is noteworthy that no nucleotide-independent alcohol dehydrogenase was found in the cell-free extract.

The NAD⁺-dependent aldehyde dehydrogenase is constitutive whilst both the NADP⁺- and the Würster's-Blue-dependent aldehyde dehydrogenases are induced during growth of the bacterium on the long-chain carbon sources. When induced, the Würster's-Blue-dependent enzyme represents approximately 80% of the aldehyde oxidizing activity present. However, the NADP⁺-dependent enzyme is induced 20-fold by the long-chain carbon sources and to a far greater extent than the Würster's-Blue-dependent enzyme which is only induced 4-fold. This disparity suggests that the NADP⁺-dependent aldehyde dehydrogenase may be more intimately associated with alkane metabolism than the nucleotide-independent enzyme.

Alcohol dehydrogenase enzymes

It seemed important to know whether the nucleotide-dependent alcohol dehydrogenase activities are carried on the same or different proteins as, for example, liver alcohol dehydrogenase shows some activity towards NADP⁺ even though it is more active with NAD⁺ (Dalziel & Dickinson, 1965). To this end, thermal stability studies of the NAD⁺- and NADP⁺-dependent alcohol dehydrogenases in crude extracts were performed. Fig. 1 shows the results obtained at 60 °C. The traces confirm the presence of distinct NAD⁺- and NADP⁺-dependent activities. The NADP⁺-dependent activity trace shows a biphasic pattern: within 5 min almost 40% of activity was lost and then over the following 90 min there was a further reduction of only 30%. The NAD⁺-dependent enzyme, on the other hand, lost 75% of activity within 15 min but then remained

Fig. 1. Temperature stability of the alcohol dehydrogenases at 60 °C: ○, NAD-dependent; ●, NADP-dependent.

almost constant over the remaining 75 min. Apart from demonstrating that the NAD⁺ and NADP⁺ activities reside on different proteins, the biphasic character of the stability traces suggests that there may well be two distinct forms of both the NAD⁺-linked and NADP⁺-linked enzymes. In the case of the NADP⁺-linked enzymes, this seems to be supported by other evidence (see below). However, as the thermal stability results were obtained using crude cell extracts one should exercise caution in interpreting them.

Following centrifugation of the cell-free extract at 200000 *g* for 1.5 h, the long-chain alcohol oxidizing activity was found entirely in the supernatant and, therefore, in the soluble fraction. The existence of separate NAD⁺- and NADP⁺-dependent alcohol dehydrogenases was confirmed by running a non-denaturing polyacrylamide gel using cell-free extract. The gel was cut longitudinally and the duplicate sample tracks were stained for activity in the presence of either NAD⁺ or NADP⁺. The gel (Fig. 2) shows that the NAD⁺-dependent activity ($R_F = 0.09$) is distinct from either of the NADP⁺-dependent alcohol dehydrogenases ($R_F = 0.315$, $R_F = 0.635$). If there are two NAD⁺-linked activities (see above) then they are not separated by this experiment. As the NAD⁺-dependent alcohol dehydrogenase represents only 10 to 15% of the alcohol oxidizing activity within the cell (Table 1) and, as this activity is also very low in absolute terms, this enzyme was not studied further.

A partial purification procedure was established for one of the NADP⁺-dependent enzymes (see Table 2*a*). Following fractionation of the cell-free extract by

Fig. 2. Non-denaturing 10% polyacrylamide gel stained for NAD(P)⁺-dependent alcohol dehydrogenases. Lane A, single band ($R_F = 0.09$) of NAD⁺-dependent alcohol dehydrogenase; lane B, two bands ($R_F = 0.315$ and 0.635) of NADP⁺-dependent alcohol dehydrogenases.

Table 2. *Specific activity and percentage total units during the purification of the NADP⁺-dependent alcohol and aldehyde dehydrogenases*

Assays were done in duplicate and were reproducible to within $\pm 5\%$; mean values are recorded.

	Specific activity (nmol min ⁻¹ mg ⁻¹)	Yield (%)	Purification factor (-fold)
<i>(a) NADP⁺-linked alcohol dehydrogenase</i>			
Cell-free extract	290	100	1
Eluate of Sephacryl S-300 column	440	22	1.5
Eluate of Reactive Red A column	12000	9	42.0
<i>(b) NADP⁺-linked aldehyde dehydrogenase*</i>			
Sephacryl S-300	600	100	1
30% Acetone supernatant (1)	280	38	
Resuspended pellet: supernatant (2)	3800	20	6.4
pellet	130	25	

* The active fractions from the Sephacryl S-300 column were pooled and precipitated with 30% (v/v) acetone. The pellet was then resuspended to give supernatant (2) containing the partially purified enzyme.

n-Aldehyde chain length

passage down the Sephacryl S-300 column, a non-denaturing polyacrylamide gel was run using the pooled

fractions containing the NADP⁺-dependent activity. When stained for activity only one band was visible and this had an $R_F = 0.314$, which corresponds to one of those shown in Fig. 2, lane B. This preparation of the enzyme was subjected to heat inactivation at 60 °C. In this case, the biphasic pattern seen previously for the NADP⁺-dependent alcohol dehydrogenase activity was not apparent. Instead, the enzyme lost 30% of its activity over 90 min. Thus only the more stable of the two NADP⁺-dependent alcohol dehydrogenases remained after the Sephacryl S-300 step. The fate of the less stable enzyme was not investigated.

The enzyme recovered from the Sephacryl S-300 step was further purified by chromatography on Reactive Red A agarose as a preliminary to a detailed substrate specificity study. The equilibrium constant of the purified enzyme was also measured at pH 7.0 and pH 8.0 using decanol as the alcohol substrate. The measured values were $K_{eq,app} = 1.01 \times 10^{-4}$ at pH 7.0 and 7.27×10^{-4} at pH 8.0. These values indicate a value for the pH-independent equilibrium constant of $K_{eq} = 8.8 \times 10^{-12}$ M. This is very close to the estimate (1.1×10^{-11} M) for the ethanol-acetaldehyde system catalysed by horse liver alcohol dehydrogenase (Bäcklin, 1958). The determination of the equilibrium constant shows that our NADP⁺-linked alcohol oxidation is a conventional alcohol dehydrogenase reaction and is not, for example, the result of the combined activity of an alcohol oxidase and an aldehyde dehydrogenase. Such a system is found in extracts of the yeast *Candida tropicalis* when grown on alkanes and it was some years before its true nature was established (Kemp *et al.*, 1988). It is important to note that no alcohol oxidase activity was detected in cell-free extracts of alkane-grown *Acinetobacter*.

The pI of the purified alcohol dehydrogenase was found to be 4.5 in an iso-electric focusing experiment. The molecular mass of the native enzyme was measured by gel filtration on a calibrated column of Sephacryl S-300 superfine. Comparison with authentic standards indicated a molecular mass of 144000 ± 5000 Da.

The K_m and V_{max} values of the NADP⁺-dependent enzyme were measured with a range of substrates varying in chain length from C₄ to C₁₄ (Table 3). The apparent K_m values of the NADP⁺-dependent alcohol dehydrogenase decreased dramatically with increasing chain length down to 5.2 µM for the C₁₄ substrate. The V_{max} values also decreased with increasing chain length. In contrast to the results with other substrates, the Lineweaver-Burk plot for butan-1-ol displayed a curved line as shown in Fig. 3 and it was not possible to measure the K_m accurately. The curved pattern displayed by the enzyme with the C₄ substrate is not due to the range of substrate concentration as the C₈ measurements were

Fig. 3. Lineweaver-Burk plot for the NADP⁺-dependent alcohol dehydrogenase using the C₄ substrate. The expected linear plot is not found with this substrate.

Table 3. Apparent K_m and V_{max} values for the alcohols used by the NADP⁺-dependent alcohol dehydrogenase

The NADP⁺ concentration was 0.25 mM. Estimates of the kinetic coefficients were generally reproducible to within $\pm 15\%$.

n-Alcohol	K_m (µM)	V_{max} (µmol min ⁻¹ mg ⁻¹)
C ₄	> 300	
C ₆	290	31.5
C ₈	120	42.0
C ₁₀	32.3	19.4
C ₁₂	4.6	6.5
C ₁₄	5.2	1.4

made over a more extended range than that used for C₄ and the usual linear pattern was obtained.

Nucleotide-dependent aldehyde dehydrogenase enzymes

As with the alcohol dehydrogenase activities, both NAD⁺- and NADP⁺-dependent aldehyde dehydrogenase enzymes were present in the crude cell-free extract. The inducibility of the NADP⁺-dependent enzyme in bacteria growing on long-chain carbon sources showed that it was a separate enzyme to that utilizing NAD⁺ as a cofactor. This was confirmed when the partially purified NADP⁺-dependent aldehyde dehydrogenase did not utilize NAD⁺. The NADP⁺-dependent enzyme represents approximately 85% of the nucleotide-dependent aldehyde oxidizing activity in extracts of cells grown on hexadecane or hexadecanol. Both the NAD⁺- and NADP⁺-dependent aldehyde dehydrogenase enzymes were present in the supernatant following a 200000 g centrifugation of the crude cell-free extract for 1.5 h.

Purification of the inducible NADP⁺-dependent enzyme was attempted. When the crude cell-free extract

was applied to a Sephacryl S-300 gel filtration column, the enzyme was eluted in the void volume of the column with a 4-fold purification (Table 2*b*). The fractions were milky in appearance. This behaviour suggested that either the enzyme is of very large molecular mass ($>10^6$ Da) and this seems rather unlikely or that it is associated with the cloudy material. *A. calcoaceticus* strain HO1-N accumulates hydrocarbon in intracellular vesicles during growth on hexadecane (Scott & Finnerty, 1976) and it appeared that the NADP⁺-dependent aldehyde dehydrogenase could well be associated with them. The fractions from the column, containing the highest enzyme activity were extracted with diethyl ether (a procedure which totally inactivated the enzyme) and the extract was analysed by gas chromatography to try to establish the nature of the material with which the enzyme was associated. The residue was found to consist of hexadecane (the growth substrate, 66%) and palmitic (16:0) and palmitoleic (16:1) acids (13% and 9%, respectively). This analysis seems to show that considerable monoterminial oxidation of hexadecane is taking place.

Various attempts were made to disrupt the association between enzyme and hydrocarbon and preserve enzyme activity. The enzyme would not bind to Reactive Red A agarose which has an affinity for NADP⁺-dependent enzymes. The effects of different solvents (C_2 to C_8 alcohols, acetone) and detergents (Triton X-100 and Brij-35) were investigated. The detergents were added to the extract to give a final concentration of 2% (v/v) and 5% (v/v). These treatments appeared to be too harsh with recoveries of only 25 and 10% of the original activity immediately following addition of the detergent. The use of the alcohols as solvents also resulted in losses of activity with no purification. Treatment of the extract with 30% (v/v) acetone appeared to be the best method for removing the hydrocarbon. This result is included in Table 2(*b*). It should be noted that there is still a very substantial amount of activity remaining in the final pellet. This activity could not be recovered with the use of detergents. For example, addition of 0.1% Triton X-100 to the pellet resulted in an immediate loss of 80% of the activity.

It seems likely that the inducible NADP⁺-dependent aldehyde dehydrogenase is present in the cell in association with the intracellular vesicles of hydrocarbon and that it is stabilized by this association. Supernatant (2) (Table 2), resulting from the acetone treatment has an opalescent appearance implying that the enzyme present is still in association with some hydrocarbon. Clearly more work is needed to resolve this problem.

Apparent K_m and V_{max} values for the purified preparation of the enzyme were measured for the various chain-length substrates (Table 4). It is clear that at very low substrate concentrations the enzyme is much more

Table 4. Apparent K_m and V_{max} values for the NADP⁺-dependent aldehyde dehydrogenase with various chain-length substrates

The NADP⁺ concentration was 0.25 mM. Estimates of the kinetic coefficients were generally reproducible to within $\pm 15\%$.

n-Aldehyde	K_m	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
C_4	3.6 mM	2.1
C_6	0.4 mM	1.2
C_8	2.1 μM	1.4
C_{10}	0.2 μM	2.3
C_{12}		>1.2
C_{14}		>1.4

effective with decanal than butanal or hexanal. This might be expected for an enzyme induced by growth on long-chain hydrocarbons. One interesting feature is that the apparent V_{max} appears to be independent of the chain length of the substrate even though the apparent K_m varies by several thousandfold. This is in contrast to the V_{max} values for the NADP⁺-dependent alcohol dehydrogenase which vary with the chain length of the substrate (Table 3). Although it is necessary to be cautious, since a complete kinetic analysis was not attempted, it seems likely that the rate-limiting step in the aldehyde dehydrogenase reaction may well be the release of NADPH from the terminal enzyme-NADPH complex. A compulsory order mechanism having this feature has been well established for mammalian liver aldehyde dehydrogenase (Hart & Dickinson, 1982, MacGibbon *et al.*, 1977).

With the long-chain substrates (dodecanal and tetradecanal) serious problems with substrate inhibition were encountered. This is illustrated in Fig. 4. Whereas decanal shows an essentially normal hyperbolic curve with perhaps a small degree of inhibition at the highest concentrations, dodecanal shows marked inhibition at all accessible concentrations. Even at the lowest concentration used (0.3 μM) the measured rate was still increasing. This behaviour presents insuperable problems to kinetic analysis. Apparent V_{max} estimates were made from the rates measured at the lowest concentration we could use. Clearly these will be minimum estimates. K_m values must be presumed to be much lower than our lowest substrate concentration.

At the concentration at which decanal was shown to be the best substrate (50 μM), butanal and hexanal were present at concentrations below their K_m values, whilst with dodecanal and tetradecanal these concentrations were very strongly inhibitory. Thus, the apparent specificity of the enzyme is dependent on the conditions chosen to investigate it (see Fig. 5). At very low concentrations (nM) the enzyme shows a strong preference for long-chain substrates but at higher concentra-

Fig. 4

Fig. 4. Activity vs substrate concentration for the NADP⁺-dependent aldehyde dehydrogenase using the C₁₂ substrate. The substrate was inhibitory at very low concentrations. Inset, expected pattern of activity vs substrate concentration for decanal.

Fig. 5

Fig. 5. Substrate specificity pattern for the NADP⁺-dependent aldehyde dehydrogenase measured with various concentrations of substrates: O, 0.3 μM; ●, 15 μM; ■, 1 mM.

Fig. 6

Fig. 6. Substrate specificity pattern for the Würster's-Blue-dependent aldehyde dehydrogenase measured in cell-free extract over a range of n-aldehydes.

Fig. 7. Semi-log plot of percentage activity of the Würster's-Blue-dependent aldehyde dehydrogenase remaining over an incubation period of 1 h. ●, 55 °C; ○, 60 °C.

Fig. 7

tions (mM) the preference shifts towards the mid chain-length aldehydes.

Nucleotide-independent aldehyde dehydrogenase

This enzyme, when induced, represents the bulk (80%) of the aldehyde oxidizing activity within the cell (see Table

1). The presence of this enzyme, which oxidizes long-chain aldehydes at the expense of the electron acceptors PMS/DCPIP and Würster's Blue, has not been reported previously in the genus *Acinetobacter*. DCPIP only functions as an electron-acceptor if PMS is also present. The PMS/DCPIP assay was not used in routine work with crude cell extracts because of the presence of a

membrane-bound NADH oxidase which affected the reduction of DCPIP.

The enzyme is found in the membrane fraction following a 200 000 *g* centrifugation for 1.5 h at 4 °C. It could be solubilized (70% yield) in a 30 min incubation at 4 °C by using 1.0% Triton X-100; other detergents (e.g. CHAPS, Brij-35, sodium cholate) were not as effective. More extended periods of extraction gave very little improvement. After 17 h in Triton X-100 75% of the enzyme was solubilized.

The substrate specificity of the enzyme in solubilized extracts was studied using C₂ to C₁₄ n-aldehydes. Fig. 6 shows that the enzyme is most active against decanal. It is noteworthy that the enzyme activity found in succinate- or acetate-grown cells also acts most effectively on the C₁₀ substrate. Of course, in this case the specific activity of the enzyme is only 25% of that seen in induced cells.

The temperature stability profile was studied at 55 and 60 °C. Fig. 7 shows first-order plots of the decline in activity. At both temperatures a biphasic pattern of activity loss was seen and the half-life of the remaining activity increased about threefold over a 20 min incubation. The reason for this is not known, but it seems that all the enzyme molecules in this crude extract may not be the same or that the denaturation process involves the generation of less active, but more stable species.

Discussion

Singer & Finnerty (1985*b*) reported the presence of three alcohol dehydrogenases in the same bacterium as has been studied here. ADH-A was induced by growth of the cells on ethanol and was essentially an NAD⁺-linked ethanol dehydrogenase. We do not find this enzyme under our growth conditions. Their other alcohol dehydrogenases had mid- to long-chain specificity: ADH-B was constitutive, NADP⁺-dependent and functioned optimally with octanol as substrate; HDH was an NAD⁺-dependent hexadecanol dehydrogenase induced 5- to 11-fold by growth of the bacterium on hexadecane or hexadecanol. Despite our repeated attempts, and using a range of potential cofactors and electron acceptors (NAD⁺, NADP⁺, PMS/DCPIP, Würster's Blue), no inducible alcohol dehydrogenase/oxidase was detected in the cells grown on hydrocarbons. Clearly, our findings are quite different from those of Singer & Finnerty (1985*b*). Jirausch *et al.* (1986), however, have described an inducible DCPIP-utilizing alcohol dehydrogenase with an activity maximally only 20% of the activity of an NADP⁺-dependent enzyme, which itself, as will be seen below, is present at much lower activities than we describe.

The NAD⁺-dependent alcohol dehydrogenase found here showed a preference for decanol while the partially purified NADP⁺-dependent enzyme showed a low apparent *K_m* of 5.2 µM for tetradecanol. Jirausch *et al.* (1986) found a constitutive NADP⁺-linked alcohol dehydrogenase in a different strain of *A. calcoaceticus* which had activity with alkan-1-ols up to decanol. Oxidation of longer-chain alcohols was not examined but presumably would have occurred.

The partially purified NADP⁺-dependent alcohol dehydrogenase studied here seems to be equivalent to that reported by Jirausch *et al.* (1986) in being constitutive and soluble. However, the level of activity that we find in crude extracts is some 17-fold greater than found by these authors. Our NAD⁺-linked enzyme may appear to be the HDH reported by Singer & Finnerty (1985*b*) but, while our enzyme is not induced, it is present in much greater amounts (up to 8-fold). The combined activities of NAD⁺- and NADP⁺-linked alcohol dehydrogenases that we find are 100-fold greater than those described by Singer & Finnerty (1985*b*).

The occurrence of such a range of alcohol dehydrogenases indicates that it would be very difficult to produce mutants of *A. calcoaceticus* which accumulate alkanols when growing on n-alkanes. It would be difficult to delete all the enzymes simultaneously.

In turning to the aldehyde dehydrogenase of this organism, the presence of the Würster's-Blue-dependent enzyme is completely novel in this genus. Nucleotide-independent aldehyde dehydrogenases have previously been reported in *Acetobacter* spp. (Ameyama *et al.*, 1981; Hommel & Kleber, 1990; Fukaya *et al.*, 1989), a *Pseudomonas* sp. (Poels *et al.*, 1987) a *Gluconobacter* sp. (Adachi *et al.*, 1978) and in *Hyphomicrobium* X (Marison & Attwood, 1980). The enzyme described here differs from those previously reported in being induced on long-chain carbon sources and having a specificity for long-chain substrates. Kleber *et al.* (1983) reported only an NADP⁺-dependent, inducible aldehyde dehydrogenase in *Acinetobacter* but could have missed the NAD⁺-dependent enzyme because of its very low activity. (By using a fluorimetric assay we were able to monitor activities much lower than can be detected by the more conventional spectrophotometric method.)

The strong induction of long-chain aldehyde oxidizing activity by growth of the bacterium on n-alkanes suggests that the aldehydes must be significant metabolites under these conditions. The lack of a corresponding increase in long-chain alcohol oxidizing activity seems to be significant but Table 1 shows that even after induction of the NADP⁺-linked aldehyde dehydrogenase the NAD⁺- and NADP⁺-linked alcohol and aldehyde dehydrogenase activities are similar. The results here do not seem to help decide whether a pathway of mono-terminal

oxidation (see route 1, Introduction) or the alternative involving hydroperoxides and per-acids (route 2) (Finnerty, 1977, 1988) is operational in n-alkane oxidation. The fact that the two aldehyde dehydrogenases are associated with different cell fractions (i.e. hydrocarbon vesicles and microsomal membranes) suggest separate roles for the two enzymes and separate destinations for the products. Whether one of these is β -oxidation and the other wax ester synthesis (Fixter *et al.*, 1986) remains to be established.

The Würster's-Blue-dependent enzyme presumably functions with some as yet unknown and probably membrane-associated electron acceptor. The action of this enzyme could perhaps allow oxidation of aldehydes without affecting $[NAD^+]/[NADH]$ or $[NADP^+]/[NADPH]$ ratios in the cell. However, a full appreciation of the significance of the Würster's-Blue-dependent enzyme requires a knowledge of its natural electron acceptor and its activity with that acceptor. So far that information is not available.

This work was funded by the European Community Biotechnology Action Programme (Grant no. 02100). We would like to thank Dr F. Meussdoerffer and Dr M. Bahn of Henkel KGaA, Dusseldorf, for their support and helpful discussion.

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