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PURIFICATION AND PROPERTIES OF A MEMBRANE-BOUND ALCOHOL DEHYDROGENASE INVOLVED IN OXIDATION OF LONG-CHAIN HYDROCARBONS BY *PSEUDOMONAS AERUGINOSA*

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SUMMARY

1. A membrane-bound alcohol dehydrogenase (alcohol:(acceptor) oxidoreductase, no EC number) has been solubilized from *Pseudomonas aeruginosa* by treatment with Triton X-100 and purified to homogeneity as judged by electrophoresis on polyacrylamide gels. (Spec. act. 6.4 units/mg protein for 1-octanol.) Aggregation of the enzyme takes place in the absence of detergent. This may be at least partly related to the lack of stability of the purified enzyme.

2. The purified enzymes does not use pyridine nucleotides (NAD or NADP) as coenzymes. No spectral evidence of the involvement of a flavin as a prosthetic group has been found. Among the artificial electron acceptors tested, only phenazine methosulfate is utilized. The product of oxidation of an alcohol is the corresponding aldehyde.

3. This alcohol dehydrogenase has a high affinity for long chain primary alcohols (K_m for 1-tetradecanol 4.5 μM). It is induced by growth on various hydrocarbons and especially by long-chain hydrocarbons (*n*-hexadecane). These properties clearly indicate its involvement in the degradation of long chain hydrocarbons in *P. aeruginosa*.

INTRODUCTION

The presence of several alcohol dehydrogenase isoenzymes (alcohol:NAD oxidoreductase, EC 1.1.1.1) in *Pseudomonas aeruginosa* has been reported previously and the properties of these enzymes have been studied[1]. Three of them utilized long-chain primary alcohols as substrates. These enzymes are present in cells grown on various carbon sources such as glucose or *n*-hexadecane. They are found in the soluble fraction of extracts prepared by ultrasonic or lysozyme treatment and their

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Abbreviations: PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol.

cytoplasmic origin is therefore likely. Gel electrophoresis experiments, however, revealed in the *P. aeruginosa* extracts the presence of a new type of alcohol dehydrogenase activity which was induced by growth on hydrocarbon substrates, particulate and requiring phenazine methosulfate (PMS) but not pyridine nucleotides for activity.

In the present paper, the purification and properties of this new alcohol dehydrogenase activity are described.

MATERIALS AND METHODS

Microorganism

The strain used, *P. aeruginosa* 196 Aa, is the one utilized in the previous work[1].

Cultures

Cells which had been transferred ten times on agar slants supplemented with 1% (w/v) glucose were subcultured in 200-ml Erlenmeyer flasks containing 20 ml of mineral medium[2] supplemented with the appropriate source of carbon (concentrations are indicated in Table II). The cells were then transferred to 2-l Fernbach flasks containing 200 ml of mineral medium supplemented with the same source of carbon and allowed to grow at 30 °C on a shaker. They were harvested (after 32 h when the carbon source was *n*-hexadecane) by centrifugation, washed with a 0.1 M potassium–sodium phosphate buffer (pH 7.4) and kept at −30 °C until use.

For purification of the alcohol dehydrogenase, larger amounts of cells were needed and the culture was carried out in a 5-l fermentor at 34 °C with high speed stirring (2000 rev./min) and forced aeration (1.0 $l \cdot min^{-1}$ per l of medium). The mineral medium[2] was modified ($MgSO_4 \cdot 7\ H_2O$ and $FeSO_4 \cdot 7\ H_2O$ were raised to 500 mg/l and 1.5 mg/l, respectively) and the carbon source was 0.8% (w/v) *n*-hexadecane. Cells were harvested in the exponential phase of growth (18 h after inoculation) and treated as described above.

Preparation and solubilization of the membranes

The procedure is summarized in Fig. 1. Unless otherwise specified all operations including purification were performed at 2–5 °C. Cells (3 g wet weight) were suspended in 6 ml of 10 mM phosphate buffer containing 5 mM $MgSO_4 \cdot 7\ H_2O$, 7 mM β-mercaptoethanol and 1 mM dithiothreitol (pH 7.3). In some cases, 100 μg of deoxyribonuclease (EC 3.1.4.5) were added.

The cells were disintegrated by sonic treatment (3 periods of 20 s at 75% of the maximal output of a Branson Sonifier) and then centrifuged at 40 000 × *g* for 60 min. The pellet was then washed with the solution used above and centrifuged in the same conditions.

The washed pellet was resuspended in 8 ml of a 10 mM phosphate buffer solution (pH 7.3) containing 15% glycerol, 7 mM β-mercaptoethanol and 1 mM dithiothreitol. A 0.9-ml volume of a 10% Triton X-100 solution was added and the membranes were gently stirred for 30 min. This homogenate was then centrifuged at 40 000 × *g* for 60 min and the supernatant was extensively dialyzed against a 20 mM phosphate buffer (pH 7.3) containing 1 mM EDTA until no reducing agent remained in the extract. This fraction (called S_2 in this paper) was used to measure the specific activity of alcohol dehydrogenase from cells grown on different carbon sources.

When the purification procedure was performed, 26 g of cells (wet weight) were used. An 84-ml volume of membrane suspension (Fraction P_2 in Table I) was obtained and was stirred for 1 h with 1% Triton X-100. This fraction was then ultracentrifuged for 60 min at 160 000 × *g*. To measure activity and protein, a small part of the supernatant was dialyzed as described above.

Enzyme assays

Problems due to the low water solubility of higher alcohols have been discussed previously[3]. However, for 1-hexadecanol a special procedure was performed: 68.4 mg were dissolved in 10 ml ethyl ether. A 100 µl volume of this solution was injected with a syringe in 500 ml of a 0.05 M Tris buffer solution (pH 7.5) previously heated at 55 °C. The ultrasonication (2 periods of 1 min at the maximal output of a Branson Sonifier) was done immediately and the solution was allowed to cool to 30 °C before use.

Alcohol dehydrogenase assay. The assay system contained 2.7 ml of 0.05 M Tris buffer (pH 7.5) with the substrate usually present as a solution (unless otherwise stated, the substrate was 2.2 mM 1-octanol), 0.1 ml of PMS (2 mg/ml) and 0.2 ml of 3 mM 2,6-dichlorophenolindophenol (DCIP). This solution was allowed to equilibrate for 5 min at 30 °C and appropriate quantities of enzyme (10–50 µl) were added to initiate the reaction.

The activity was assayed by measuring the decrease in absorbance of DCIP at 578 nm with a recording Eppendorf spectrophotometer at 30 °C.

A unit is defined as the amount of enzyme which catalyzes the conversion of 1 µmole of substrate per min at 30 °C. The calculations are based on a molar extinction coefficient for DCIP at 578 nm of 14.5 $mM^{-1} \cdot cm^{-1}$.

NAD-dependent aldehyde dehydrogenase assay. The assay system contained 2.8 ml of 0.05 M Tris buffer (pH 7.5) with the substrate (1.1 mM octanal) present as a solution, 1 mM NAD and appropriate quantities of enzyme in a final volume of 3 ml. The reaction was initiated by addition of enzyme. The activity was assayed by measuring the increase of absorbance at 334 nm resulting from NAD reduction. The equipment used and the definition of enzyme unit are the same as above.

Protein assays

Protein assays were done using the method of Folin[4] with serum albumin as a standard.

Analysis of products by gas–liquid chromatography

A flame-ionization Aerograph 1520 chromatograph was used. The products were separated on a 5 ft column packed with 15% polyethylene glycol terephtalate ("Free Fatty Acids Phase") on chromosorb W. from Varian Aerograph at a temperature of 180 °C. The incubation was done in 3-ml cuvettes with or without 1 mM NAD in the conditions described for alcohol dehydrogenase assay. Aliquots of 1 µl were withdrawn at different times and immediately injected into the column of the chromatograph.

Chromatographic techniques

The methods utilized have been described in the preceding paper[1].

Analytical gel electrophoresis

The conditions described previously[1] were slightly modified as follows: the gels contained 0.05% Triton X-100. The developing solution used for staining for activity contained 3 ml of 0.05 M Tris buffer (pH 7.5) with 2.2 mM 1-octanol, 0.12 ml of PMS solution (2 mg/ml) and 0.15 ml of tetrazolium red (10 mg/ml). Purple spots of the reduced formazan were visible less than 30 min after incubation in the dark at 37 °C was started. For protein staining, Coomassie brilliant blue was used[5].

Reagents

The origin of the various alcohols, diethylaminoethyl cellulose, hydroxyapatite and NAD was the same as previously indicated[1]. Diethoxymethane was from Fluka, aminopterin from Serva and pyocyanine from K and K laboratories.

RESULTS

Localization of the long-chain alcohol dehydrogenases

The homogenate obtained by ultrasonic treatment from cells grown with *n*-hexadecane as a carbon source was fractionated by differential centrifugation, according to the procedure given in Fig. 1. This homogenate contained a NAD(P)-independent, long-chain alcohol dehydrogenase, functioning with PMS as a cofactor.

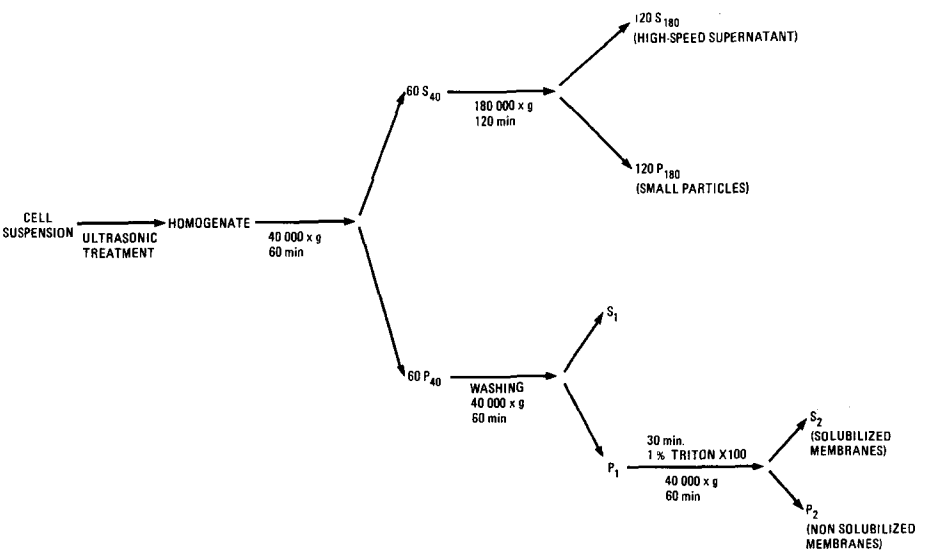

Fig. 1. Scheme of solubilization of the PMS-dependent alcohol dehydrogenase.

From the results given in Table I it can be concluded that most of the activity is located in particles sedimenting at 40 000 × *g*. By comparison, after the same treatment, 96% of the NADP-dependent alcohol dehydrogenase I (ref. 1) was present in the supernatant at 40 000 × *g*.

TABLE I

DISTRIBUTION OF THE PMS-DEPENDENT LONG-CHAIN ALCOHOL DEHYDROGENASE ACTIVITIES DURING THE SOLUBILIZATION PROCEDURE

Activities are given in percentage of the activity for the homogenate.

Fraction	*Symbol*	*Activity (%)*
Homogenate	—	100
Low speed supernatant	60 S_{40}	13
Membrane suspension	P_1	87
High-speed supernatant	120 S_{180}	9
Small particles	120 P_{180}	3
Solubilized membrane fraction	S_2	34
Non solubilized membrane fraction	P_2	25

Regulation of the long-chain alcohol dehydrogenase

The specific activities of the solubilized membranes (S_2) have been measured for cells grown on different substrates (Table II). The results show clearly that this enzyme is inducible. It is interesting to note that the specific activity is greater for cells grown on *n*-hexadecane than for cells grown on *n*-heptane. We have verified that the K_m values for various alcohols were identical in enzyme preparations from cells grown on these two carbon sources. Another point is that succinate seems to be a strong repressor, in fact better than glucose. Such a result has already been reported for other degradative enzymes in *P. aeruginosa* (ref. 6).

TABLE II

INDUCTION OF THE PMS-DEPENDENT ALCOHOL DEHYDROGENASE

Growth substrate	*Spec. act. (units/mg) of solubilized membranes (S_2)*
Sodium succinate (50 mM)	0.005
Glucose (85 mM)	0.029
Sodium malonate (40 mM)	0.013
Sodium malonate (40 mM) + diéthoxymethane (0.4%)	0.054
n-heptane (0.4%)	0.140
1,10-decanediol (10 mM)	0.198
n-hexadecane (0.5%)	0.760

The experiment with malonate plus diethoxymethane (Table II) was done because of the finding of Van Eyk and Bartels[7] that diethoxymethane was an inducer for alcane hydroxylase of cells grown on malonate. We have confirmed this result for our strain (unpublished results). It seems, however, that in the same conditions, the PMS-dependent long-chain alcohol dehydrogenase is very poorly induced.

Choice of the pH for the enzyme assay

The assay was performed at pH 7.5 as described in Materials and Methods. This pH was chosen in spite of the higher activity observed at more basic pH values (1.5-fold greater at pH 9.0 than at pH 7.0), because it was thought to be representative

of physiological conditions and also because some oxidation of DCIP was observed in the control cuvette (incubation without alcohol) at pH values above 7.5. At pH 7.5, there was no residual activity in the control cuvette for Fraction S_2 and the reaction proceeded at a linear rate during the time of the assay. For the fractions obtained before the solubilization step a residual activity was found in the control cuvettes.

Properties of the fraction obtained by solubilization of the membranes (S_2)

This fraction was used to identify the product of the reaction studied. As shown in Fig. 2, incubation of alcohol and enzyme with PMS and DCIP as cofactors led to a single product which was the corresponding aldehyde. However, when 1 mM NAD was added to the incubation mixture, both the corresponding aldehyde and acid were detected (Fig. 2). This result suggested the presence of one or more NAD-dependent aldehyde dehydrogenases in the extract, and this hypothesis was confirmed by demonstrating by spectrophotometry that the extracts utilized catalyzed the reduction of NAD with octanal as substrate. NADP could not replace NAD as the coenzyme.

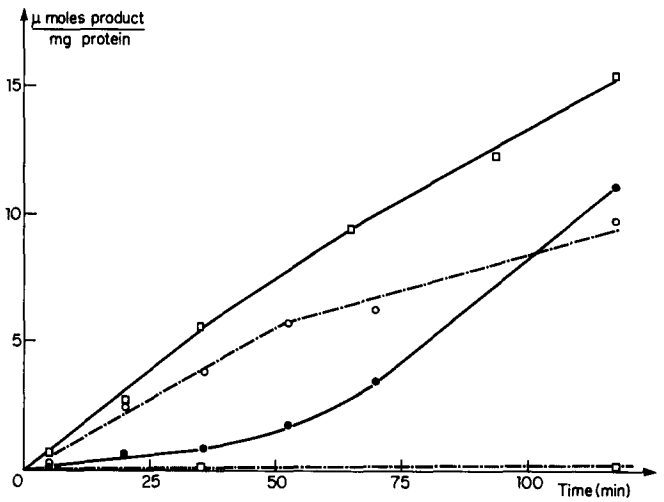

Fig. 2. Products formed by incubation of the solubilized membrane fraction with 1-octanol. The incubation mixture contained 2 mM 1-octanol. The reaction was initiated by the addition of 0.108 mg protein of a solubilized membrane fraction (S_2) from *n*-heptane-grown cells. □—□, aldehyde formed (incubation without NAD); ○—·—○, acid formed (incubation with NAD); □—·—□ acid formed (incubation without NAD); ●—●, aldehyde formed (incubation with NAD).

The reasons for the kinetics of aldehyde and acid production in the presence of NAD (Fig. 2) are not known, as incubation of aldehyde dehydrogenase for 1 h at 30 °C with 1 mM octanal did not change its activity as measured by the spectrophotometric determination of NAD reduction. Moreover, the same pattern of products formation was observed when the incubation mixture contained catalase (150 units/ml) to eliminate H_2O_2 produced by the reoxidation of PMS. In addition, it has been found that 1 mM octanoic acid was not inhibitory. The results, however, suggest

inactivation of aldehyde dehydrogenase which may result because of the particular assay conditions, such as the use of PMS which has recently been reported to produce superoxide ion O_2^- upon reoxidation[8].

Purification procedure

As cells grown on *n*-hexadecane yield extracts with the highest specific activity for this enzyme, this carbon source was chosen to grow the cells used for the purification of the enzyme. The activities and yields are summarized in Table III.

TABLE III

PURIFICATION OF THE PMS-DEPENDENT LONG-CHAIN ALCOHOL DEHYDROGENASE

As illustrated by this table an important loss of activity occurred during concentration and storage between two purification steps.

Preparation		*Volume (ml)*	*Total activity (units)*	*Spec. act. (units/mg)*	*Overall yield (%)*	*Overall purification*
Homogenate		66	725	0.230		
Treatment with Triton X-100 (ultra centrifugation supernatant)		71	226	0.505*	31.2	2.3
DEAE-cellulose eluate		70	182	2.35	25.5	10.2
Hydroxyapatite	charge	30	132	1.70	18.2	
	eluate maximum	50	100	4.0	13.8	17.4
	fraction	10	32.5	5.6	4.5	24.3
Sephadex G-200	charge	1.2	18.1	3.0		
	eluate maximum	22	9.35	3.83		
	fraction	3	2.01	6.4	0.28	27.8

* The lower activity found in the solubilized membrane fractions used for purification purposes (compare with activity in Table II) probably results from the different conditions used for their preparation (see Materials and Methods).

Stability. The solubilized fraction (S_2) loses less than 5% of activity in 8 days when stored at −30 °C with or without reducing agents. However, the stability decreases in the course of the purification: the eluate from DEAE-cellulose stored at −30 °C with 0.05% Triton X-100 loses 25% of its activity in 48 h. In some cases, substrate (0.4 mM 1-octanol) and 2 mM dithiothreitol were added after hydroxyapatite chromatography to improve the stability. Because of this unstability, the whole procedure had to be carried out as rapidly as possible.

DEAE-cellulose chromatography. The solubilized membranes (S_2) obtained as described above were dialyzed against a 1 mM potassium phosphate buffer (pH 7.4) containing 0.05% Triton X-100, 15% glycerol and 1 mM EDTA. The preparation (70 ml) was applied to a DEAE-cellulose column that had been equilibrated with the same buffer, as indicated in the legend of Fig. 3. After the sample had been adsorbed on the top of the column, 30 ml of the same buffer were added and elution was started with a convex phosphate gradient in a concentration range from 1 to 250 mM phosphate. The results are shown in Fig. 3. The bulk of alcohol dehydrogenase activity was eluted at phosphate concentrations between 45 and 60 mM phosphate. This step resulted in a 4.6-fold purification and an 81% recovery. As shown in Fig. 3 an

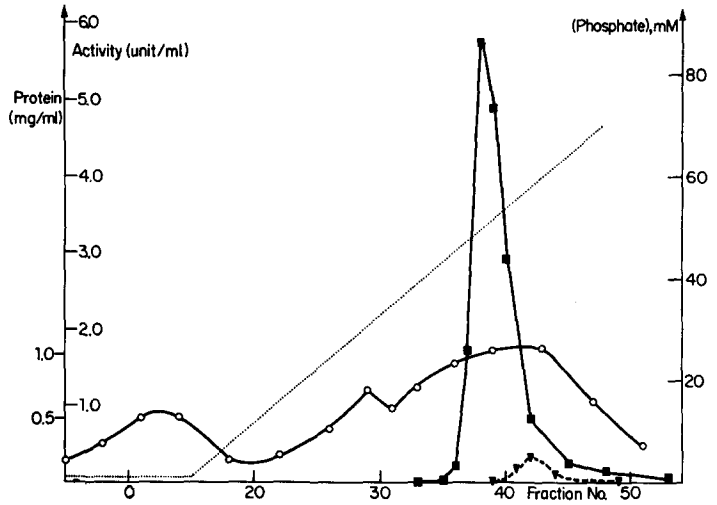

Fig. 3. DEAE-cellulose chromatography of solubilized membranes (S_2). 222 units of PMS-dependent alcohol dehydrogenase in a volume of 70 ml containing 6.3 mg of protein per ml were charged on a 2.54 cm × 39 cm DEAE-cellulose column. 10-ml fractions were collected. The elution rate was 30 ml/h. ○—○, protein concentration (mg/ml); ■—■, PMS-dependent alcohol dehydrogenase activities (units/ml); ▼- - -▼, NAD-dependent aldehyde dehydrogenase activi- (units/ml); ···, calculated phosphate gradient (mM).

aldehyde dehydrogenase was eluted in the same range of phosphate concentrations.

Hydroxyapatite chromatography. Fractions 37 to 42 from DEAE-cellulose chromatography (Fig. 3) were combined and dialyzed several times against a 1 mM phosphate buffer (pH 7.3) containing 0.05% Triton X-100, 15% glycerol and 1 mM

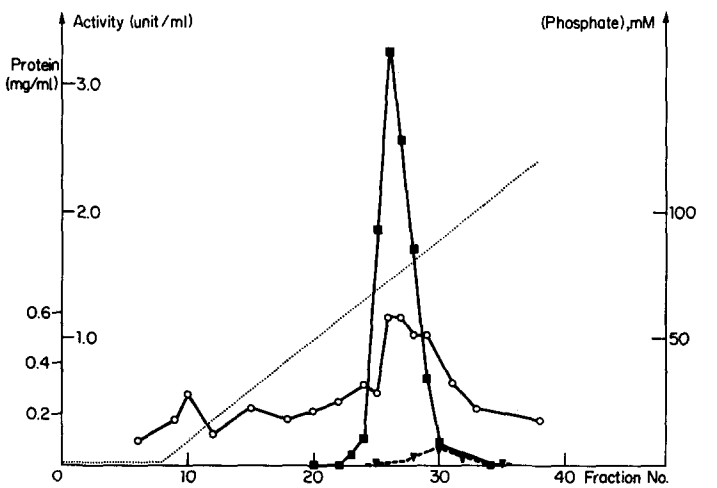

Fig. 4. Hydroxyapatite chromatography. 132 units of PMS-dependent alcohol dehydrogenase (Fractions 37 to 42 from DEAE-cellulose chromatography eluate) in a volume of 30 ml, containing 2.6 mg of protein per ml, were charged on a 2.54 cm × 29.5 cm hydroxyapatite column. 10-ml fractions were collected. The elution rate was 30 ml/h. ○—○, protein concentration (mg/ml); ■—■, PMS-dependent alcohol dehydrogenase activities (units/ml); ▼- - -▼, NAD-dependent aldehyde dehydrogenase activities (units/ml); ···, calculated phosphate gradient (mM).

EDTA. The resulting solution (30 ml) was applied to a hydroxyapatite column previously equilibrated with the same buffer. The column was washed with 40 ml of equilibration buffer and then elution with a convex gradient ranging from 1 to 400 mM was started. Results are shown in Fig. 4.

The bulk of the alcohol dehydrogenase was eluted at a phosphate concentration ranging from 65 to 90 mM, resulting in a 2.35-fold purification and a 77% recovery.

The NAD-dependent aldehyde dehydrogenase was slightly more retarded than the alcohol dehydrogenase but the two enzymes were not completely separated.

Sephadex G-200 chromatography. Fraction 26 from hydroxyapatite chromatography which was devoid of aldehyde dehydrogenase activity, was concentrated to 1.2 ml by ultrafiltration. This sample was loaded on a Sephadex G-200 column which had been equilibrated with a 0.1 M phosphate buffer (pH 7.4) containing 0.05% Triton X-100 and 1 mM EDTA. Results are shown in Fig. 5. This step resulted in a 1.28-fold purification and 52% recovery. The highest specific activity of the alcohol dehydrogenase was 6.4 units/mg protein.

The ratio V_e/V_0 obtained for the enzyme in these conditions is 1.35. This suggests a molecular weight in the range of 250 000–350 000 (ref. 9).

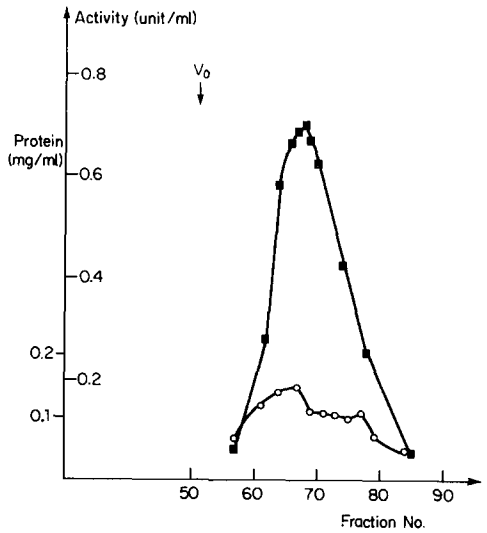

Fig. 5. Chromatography on Sephadex G-200. 18.1 units in a volume of 1.2 ml containing 5 mg of protein per ml were charged on a 2.15 cm × 39.5 cm Sephadex G-200 column. 1-ml fractions were collected. The elution rate was 22 ml/h. ○—○, protein concentration (mg/ml); ■—■, PMS-dependent alcohol dehydrogenase activities (units/ml). The dead volume (V_0) was measured with blue dextran in the same conditions as used for chromatography of the enzyme.

Acrylamide gel electrophoresis

Staining for activity. For the S_2 fraction only one band of activity was detected on acrylamide gel. This band was identical for all the alcohols tested (from 1-butanol to 1-tetradecanol). The R_F was 0.27 ± 0.02.

Staining for proteins. The most active fractions eluted from Sephadex G-200 showed only one protein band. However, in some cases, a very light trailing could

be seen behind this protein band. After six days of storage at −30 °C new very slowly migrating bands appeared on the upper part of the gel.

Properties of the long-chain alcohol dehydrogenase

Assay of activity of the alcohol dehydrogenase with various electron acceptors. Table IV shows that this alcohol dehydrogenase is specific for PMS. There is no activity with NAD or NADP whatever the pH (7.5 or 9).

TABLE IV

UTILIZATION OF VARIOUS ELECTRON ACCEPTORS BY THE LONG-CHAIN ALCOHOL DEHYDROGENASE

The preparation used was an eluate from hydroxyapatite chromatography. The activity was determined by measurement of the aldehyde formed by gas chromatography and, where possible, by spectrophotometry. The agreement between these two methods was quite good (they gave the same results for the assay with PMS + DCIP). The standard assay conditions were used. The substrate was $1 \cdot 10^{-4}$ M 1-decanol.

Electron acceptor	*Activity* (%)*
None	1
PMS (0.2 mM)	100**
PMS (0.2 mM) + DCIP (0.2 mM)	100***
DCIP (0.2 mM)	9***
Pyocyanine (0.2 mM)	5
Pyocyanine (0.2 mM) + DCIP (0.2 mM)	5***
Cytochrome *c* (0.5 mg/ml)	6***

* Activities are given as percentage of that obtained in the assay with PMS.
** Spec. act.: 1.03 units/mg with $1 \cdot 10^{-4}$ M 1-decanol.
*** The more precise determinations obtained by spectrophotometry are given.

Measurements with a Clark oxygen electrode have shown that this enzyme does not present the properties of an oxidase. (No O_2 consumption was found when the enzyme was incubated at 25 °C in a 0.05 M Tris buffer (pH 7.5) containing 2 mM 1-octanol.) In view of the remarkable specificity for PMS of this alcohol dehydrogenase (even pyocyanine, also a phenazine pigment, is not an electron acceptor for this enzyme), it appeared appropriate to designate this enzyme PMS-dependent long-chain alcohol dehydrogenase. In spite of the fact that phenazine pigments are known to be produced by *P. aeruginosa* (ref. 10), this denomination is not intended to imply the identification of PMS with the physiological electron acceptor for this enzyme.

Measurement of kinetic constants. Apparent K_m and V values were determined with different alcohols and are given in Table V. Like alcohol dehydrogenase I (ref. 1) the PMS-dependent long-chain alcohol dehydrogenase shows lower K_m values for the long-chain alcohols but here the values are even lower, (by a factor of about 20 in the case of 1-dodecanol). As for alcohol dehydrogenase I, the maximal velocities decrease with the length of the alcohols. In the case of 1-hexadecanol, the low water solubility of this alcohol made the determination of initial velocities more difficult. However, by performing the experiment in the presence of 1 mM NAD, reliable spectrophotometric readings were obtained because of the presence of a NAD-dependent aldehyde dehydrogenase in the S_2 fraction used. It has been verified in the case of 1-dodecanol and 1-tetradecanol that K_m values were identical whether they were measured in the presence or the absence of NAD. The maximal velocities

TABLE V

KINETIC CONSTANTS OF THE PMS-DEPENDENT LONG-CHAIN ALCOHOL DEHYDROGENASE

Kinetic constants (apparent K_m and V values) were measured at a PMS concentration of 0.2 mM. This concentration is saturating for PMS. Maximal velocities are given as percentage of the maximal velocity for 1-octanol. The activity with ethanol was very low (at 1 M concentration it was 3% of the maximal activity for 1-octanol).

Substrates	K_m *(mM)*	V *(%)*
1-Butanol	420	140
1-hexanol	33	140
1-Octanol	0.4	100
1-Decanol	0.02	100
1-Dodecanol	0.0045	85
1-Tetradecanol	0.0045	85
1-Hexadecanol	0.0055	25

measured were, as expected, higher (1.7-fold in the case of 1-dodecanol and 1-tetradecanol) when the determination was carried out in the presence of NAD.

Studies with inhibitors. In Table VI, the results obtained with a variety of inhibitors are presented. No metal chelating agent greatly affected the activity. The only thiol reagent among those tested which inhibited the enzyme was *p*-hydroxymercuribenzoate. Aminopterin, which inhibits some pteridine-dependent enzymes[16] did not affect the activity of this enzyme under the conditions used.

Spectra. The ultraviolet and visible spectra of the purified enzyme are presented in Fig. 6. Their characteristics do not support the idea of the existence of a flavin or a pyridine nucleotide as a bound prosthetic group for this enzyme. The interpretation of the ultraviolet spectrum is complicated because of the presence of Triton X-100 which is necessary to prevent aggregation of the enzyme (see Discussion) and absorbs in this region.

TABLE VI

EFFECT OF VARIOUS INHIBITORS ON PMS-DEPENDENT ALCOHOL DEHYDROGENASE

The preparation used was a solubilized fraction (S_2) from *n*-hexadecane grown cells. The inhibition was measured either immediately or after the indicated period of preincubation with the inhibitor in the absence of substrates. The alcohol was 0.4 mM 1-octanol.

Inhibitor	*Concentration (mM)*	*Incubation time (min)*	*% Inhibition*
EDTA	10	0	0
	10	40	0
1-10 Phenantroline	1	0	5
	1	40	37
α,α′-Dipyridyl	1	40	6
Iodoacetamide	1	0	0
	1	40	12
Sodium iodoacetate	1	40	14
p-Hydroxymercuribenzoate	1	0	33
	1	40	100
Aminopterin	1	0	0

DISCUSSION

The experiments reported here have established the existence of NAD(P)-

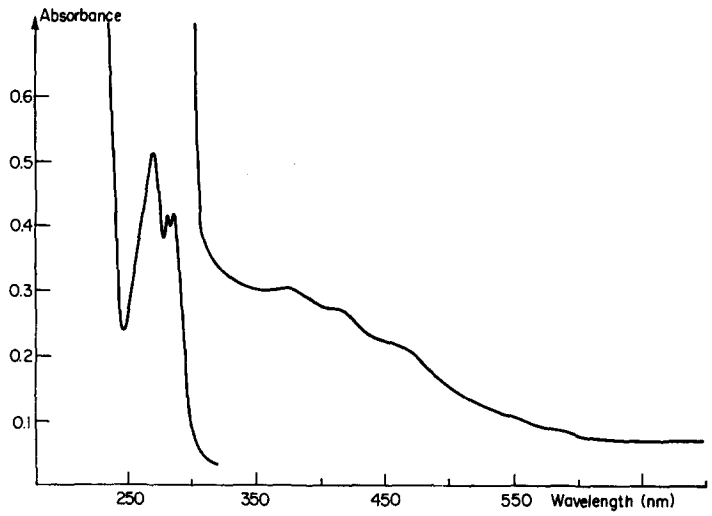

Fig. 6. Ultraviolet and visible spectra of the purified PMS-dependent long-chain alcohol dehydrogenase. The concentrations used were 0.5 mg/ml for the ultraviolet spectrum and 5 mg/ml for the visible spectrum. The specific activity was 5.6 units/mg. The sample and reference cuvettes contained the medium used for hydroxyapatite chromatography. No important change was observed in the visible region when substrate (0.4 mM 1-octanol) or dithionite was added to reduce the enzyme.

independent alcohol dehydrogenase, which utilizes PMS as an electron acceptor. The kinetic properties of this enzyme, as well as its regulation pattern, clearly indicate the involvement of this alcohol dehydrogenase in the metabolism of long-chain hydrocarbons.

The presence of a NAD(P)-independent alcohol dehydrogenase induced by growth on hydrocarbons has already been reported in *P. aeruginosa* (ref. 11) and it is possible that this enzyme is identical to that described here. The comparison actually is difficult as no purification was done in the work just mentioned. However, some differences are apparent, especially the lack of specificity of this enzyme for PMS and its inhibition by excess substrate.

The localization of the present enzyme in the cell membrane is clearly established. Some solubilization takes place during the sonic treatment but the study of the preparations obtained in this way was complicated as they gave rise to several activity bands on gel electrophoresis and several peaks of alcohol dehydrogenase activity by chromatography on DEAE-cellulose columns (in addition to the alcohol dehydrogenase isoenzymes previously studied[1]). On the contrary, membrane preparations solubilized with Triton X-100 gave a single sharp activity band on gel electrophoresis and one peak of alcohol dehydrogenase activity on DEAE-cellulose (Fig. 3) and this was a reason for the adoption of this solubilization procedure.

In addition, it has been found that upon removal of Triton, aggregation of the enzyme takes place. This was shown by applying a purified fraction to a Sephadex G-200 column in conditions identical to those used for the last purification step except for the omission of Triton in the elution buffer. The peak of activity in these conditions was completely excluded from the gel. These properties may explain the characteristics of the preparations obtained by sonic treatment and suggest the

existence on the outer surface of the enzyme of hydrophobic regions which on removal of the detergent, may interact, resulting in aggregation. They are in line with the membrane localization of this enzyme.

The nature of the physiological electron acceptor used by the enzyme described here is unknown. It is clear that the properties of a solubilized enzyme may be affected by the solubilization procedure and thus do not give a complete picture of the situation *in vivo*. However, the utilization of an electron acceptor with a higher reduction potential such as PMS ($E_0' = 0.080$ V), in preference to NAD, may have a physiological value as it may facilitate the oxidation of long-chain alcohols, which are probably present at quite low concentrations, by displacing the thermodynamic equilibrium towards the formation of aldehyde. This argument does not apply to aldehyde dehydrogenase because the oxidation of an aldehyde to an acid is thermodynamically much more favorable than that of an alcohol to an aldehyde.

Many examples of pyridine nucleotide-independent dehydrogenases have been reported. Among them, several enzymes of bacterial origin, in particular from *Pseudomonas* species are worth mentioning[12–15]. All these enzymes are localized in the cytoplasmic membrane and catalyze the dehydrogenation of an alcoholic group. They are assayed with artificial electron acceptors. Among those which have been purified, methanol dehydrogenase[14] is specific for PMS and the involvement of a pteridine cofactor has been suggested. It is active with higher alcohols (up to 1-undecanol) and its sensitivity to inhibitors presents some common points with the enzyme described here.

The case of malate dehydrogenase[13] also is interesting because a special effort was made to elucidate the nature of the physiological electron acceptors utilized by this enzyme. It was shown that a combination of FAD, quinone (Coenzyme Q_9) and phospholipid restored the activity of the purified enzyme.

More information regarding the nature of the physiological electron acceptors of this long-chain alcohol dehydrogenase appears desirable but some improvement in the stability of the purified enzyme would be beneficial to these studies.

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