Identification and Characterization of a Nicotinamide Adenine Dinucleotide-Dependent *p*-Hydroxybenzyl Alcohol Dehydrogenase from *Rhodopseudomonas acidophila* M402

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A nicotinamide adenine dinucleotide-linked *p*-hydroxybenzyl alcohol dehydrogenase activity was demonstrated in cells of *Rhodopseudomonas acidophila* M402 grown on *p*-hydroxybenzyl alcohol as a major carbon source under anaerobic-light conditions. The enzyme was purified to homogeneity on polyacrylamide gel electrophoresis. The molecular weight of the enzyme was estimated to be 27,000 dalton by gel filtration and the isoelectric point was pH 7.4. This dehydrogenase requires NAD⁺ as an electron acceptor and has broad specificity for aromatic alcohols. This enzyme is also capable of oxidizing aliphatic primary alcohols with NAD⁺.

While this enzyme was induced by *p*-hydroxybenzyl alcohol, the final preparation showed the highest affinity for vanillyl and cinnamyl alcohols rather than *p*-hydroxybenzyl or benzyl alcohols. Para-derivatives (methyl, methoxy, nitro or chloro) of benzyl alcohol also underwent the dehydrogenation reaction. Moreover, the enzyme was also active on *m*-derivatives (hydroxy, methyl, methoxy, nitro or chloro) of benzyl alcohol, 39 to 55% of the activity seen for *p*-hydroxybenzyl alcohol. The most interesting characteristic of this dehydrogenase is its wide affinity range for aliphatic alcohols: 1-butanol, 1-pentanol, 3-methyl-1-butanol, 1-hexanol, 1-heptanol, 1-octanol and 1-nonanol. This substrate specificity resembles that of dye-linked alcohol dehydrogenase (PMS-linked vanillyl alcohol dehydrogenase) from the same organism, but the two enzymes differ in their mode of induction, electron acceptor requirement, ammonium requirement for activity, pH optima, molecular weight and other aspects. A typical NAD⁺-dependent alcohol dehydrogenase oxidizes ethanol and other primary alcohols, and acetaldehyde, but not methanol or aromatic alcohols such as benzyl alcohol, hydroxybenzyl alcohol and vanillyl alcohol. Therefore, the *p*-hydroxybenzyl alcohol dehydrogenase reported in this paper is a new type of alcohol dehydrogenase which is different from hitherto reported alcohol dehydrogenases.

Many aerobic bacteria have been isolated as potent utilizers of a variety of aromatic compounds. Research on the biodegradation of aromatic compounds were conducted from environmental, ecological and biochemical aspects. However, these studies can be put into two categories: First, the biodegradation of aromatic compounds carried out mainly by aerobic microorganisms under aerobic conditions. Second, aromatic acids such as benzoate, protocatechuate, gentisate and salicylate were selected as substrates for these microorganisms. Consequently, the biodegradation pathway of aromatic acids under aerobic conditions is well established. Reductive

biodegradation of aromatic compounds has also been studied with some anaerobic bacteria, e.g. benzoate and eleven aromatic compounds under methanogenic conditions¹⁻³⁾ or with anaerobic nitrate reduction.⁴⁾ Several species of purple nonsulfur phototrophic bacteria, the *Rhodospirillaceae* family, are able to grow at the expense of a single aromatic compound as the sole carbon source. *Rhodopseudomonas palustris*, *Rp. capsulata*, *Rp. spheroides*, *Rp. gelatinosa* and *Rhodomicrobium rubrum* showed growth with benzoate as carbon source anaerobically in the light.^{5,6)} But no papers have appeared on the utilization ability for aromatic aldehydes or alcohols under anaer-

obic conditions.

In previous studies, we reported that newly isolated *Rhodopseudomonas acidophila* M402 was able to utilize vanillyl alcohol aerobically in the dark and also anaerobically in the light.⁷⁾ A new dye-linked vanillyl alcohol dehydrogenase was found in the extracts of cells grown aerobically on vanillyl alcohol.^{8,9)} Studies on the utilization of aromatic alcohols were extended to benzyl and hydroxybenzyl alcohols. These alcohols were capable of supporting bacterial growth under anaerobic-light conditions. Enzyme systems for catabolism of these alcohols were analyzed and a new enzyme, *p*-hydroxybenzyl alcohol dehydrogenase, was found in cells anaerobically grown on *p*-hydroxybenzyl alcohol in the light. This paper describes the purification and properties of NAD⁺-*p*-hydroxybenzyl alcohol dehydrogenase from *R. acidophila* M402 and also discusses the correlation between this enzyme and the previously found PMS-alcohol (non-specific) dehydrogenase.⁹⁾

MATERIALS AND METHODS

Cultivation of the organism. *Rhodopseudomonas acidophila* M402 was grown on a medium composed of (g/l) KH₂PO₄ 0.5, K₂HPO₄ 1.0, NH₄Cl 0.5, MgSO₄·7H₂O 0.12, NaCl 0.1, CaCl₂ 0.05 and FeCl₃ 0.005 under anaerobic-light conditions. An aromatic alcohol was supplemented at 5 mM as the sole carbon source. The pH was adjusted to 7.0 before autoclaving. Cultivation was carried out in a 10-liter jar fermentor (NBS MF-114) equipped with an automatic pH controller. The temperature was kept at 30°C. The culture was maintained under anaerobic conditions. The head space of the jar was filled with nitrogen gas and the medium was bubbled with sterilized nitrogen gas as described in a previous paper.¹⁰⁾ The culture was illuminated at 3,000 lux with 40W tungsten bulbs.

Cell-free extracts. Cells were harvested by centrifugation after 90 hrs culture. Cells were washed twice with 500 ml of chilled 50 mM Tris-HCl buffer, pH 8.0. The yield of cells was 9.4~15.2 g wet weight from 10-liters of medium. The washed cells (wet weight: 15.2 g) were suspended in 130 ml of the same buffer and disrupted by ultrasonic oscillation (20 kHz) for 4 min under ice-cooling. The cell-free extracts were obtained after centrifugation at 16,000 × *g* for 20 min.

Assay of aromatic alcohol dehydrogenase activity. The standard assay for NAD⁺-linked dehydrogenase activities was carried out with a reaction mixture containing the following components: 0.5 ml of 0.05 M glycine buffer, pH 9.5; 0.05 ml of 0.05 M NAD; 0.1 ml of 0.05 M aromatic alcohol and enzyme solution in a final volume of 1.0 ml. The reaction was initiated by the addition of the enzyme. Cuvettes were thermostated at 30°C. The reaction rate of NADH formation was measured at 370 nm instead of 340 nm. Absorption at 340 nm of newly formed aromatic aldehyde, e.g. *p*-hydroxybenzaldehyde, interfered with the enzyme assay measurement at 340 nm. The molecular extinction coefficient of *p*-hydroxybenzaldehyde at 340 nm was calculated to be 2.1×10^4 /mol·cm at pH 9.5. A molecular extinction coefficient of NADH at 370 nm of 2.8×10^3 /mol·cm was used.¹¹⁾ One unit of enzyme was defined as the amount of enzyme which converted 1 μmol of hydroxybenzyl alcohol to hydroxybenzaldehyde per min. Specific activity was expressed as micromoles of NADH reduced per min per mg of protein.

Polyacrylamide gel electrophoresis. This was performed as reported in the previous paper.⁹⁾ Dehydrogenase activity on various alcohols was detected in the gels by the previously described procedure. Stained bands were recorded by scanning at 580 nm and relative mobilities of the bands were calculated.

Assays of aromatic alcohols, aldehyde and acids. *p*-Hydroxybenzyl alcohol and its intermediates, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid, in the medium were identified and assayed by reverse-phase high performance liquid chromatography.⁷⁾ Vanillin was used as an internal standard.

Isoelectric focusing. Isoelectric focusing was performed with 1% (w/v) carrier Ampholite, pH 3~10, at a constant voltage of 450 V for 60 hr as described in the previous paper.⁹⁾

Molecular weight determination. This was carried out with a column of Sephacryl S-300 by the method of Andrews.¹²⁾

Chemicals. All aromatic and aliphatic alcohols were of reagent grade and purchased from Nakarai Chemicals Ltd. Vanillyl alcohol was purchased from Tokyo Kasei Kogyo Co. and purified by recrystallization from chloroform solution. Sephacryl S-300 was a product of Pharmacia Fine Chemicals.

RESULTS

Induction of aromatic alcohol dehydrogenases

Among forty strains of the Rhodospirillaceae family tested, *Rhodopseudomonas acido-*

TABLE I. GROWTH ON HYDROXYBENZYL AND BENZYL ALCOHOLS UNDER ANAEROBIC-LIGHT CONDITIONS

Culture were grown for 170 hr.

Strain		Bacterial growth ($A_{650\text{ nm}}$) on		
		<i>p</i> -Hydroxybenzyl alcohol	<i>m</i> -Hydroxybenzyl alcohol	Benzyl alcohol
<i>Rhodopseudomonas acidophila</i>	M402	1.23	0.70	1.53
<i>Rhodopseudomonas acidophila</i>	10050	0.18	0.16	0.20
<i>Rp. palustris</i>	3-1	0.93	0.50	0.96
<i>Rp. palustris</i>	7-1	0.86	0.50	0.41
<i>Rp. palustris</i>	8-1	0.69	0.45	0.82
<i>Rp. palustris</i>	8-6	0.80	0.65	0.91
<i>Rp. palustris</i>	50	0.89	0.79	1.43
<i>Rhodomicrobium</i> sp.	1	0.57	0.53	0.53
<i>Rhodomicrobium</i> sp.	2	0.41	0.42	0.46
<i>Rhodomicrobium</i> sp.	3	0.76	0.91	0.90

TABLE II. INDUCTION OF HYDROXYBENZYL AND BENZYL ALCOHOL DEHYDROGENASES

C-Source (5 mM)	Protein in extracts (mg)	Cofactor	Dehydrogenase activities (milliunits) on						
			<i>p</i> -Hydroxy- benzyl alcohol	<i>m</i> -Hydroxy- benzyl alcohol	<i>o</i> -Hydroxy- benzyl alcohol	Benzyl alcohol	Benz- aldehyde	Vanillyl alcohol	Vanillin
<i>p</i> -Hydroxybenzyl alcohol	58.9	NAD ⁺ ^a	530	40	ND	300	80	60	30
		PMS ^b	ND ^c	ND	ND	ND	ND	ND	ND
<i>m</i> -Hydroxybenzyl alcohol	52.1	NAD ⁺	10	5	30	40	30	ND	ND
		PMS	ND	ND	ND	ND	ND	ND	ND
Benzyl alcohol	62.2	NAD ⁺	290	250	200	620	250	60	4
		PMS	ND	ND	ND	ND	ND	ND	ND
Vanillyl alcohol	20.9	NAD ⁺	126	ND	ND	56	ND	252	230
		PMS	14	8	ND	ND	ND	16	56

^a Activity was measured at pH 9.5.

^b Activity was measured at pH 7.5.

^c ND, not detected.

phila M402 showed significant growth on three aromatic alcohols, *p*-hydroxybenzyl, *m*-hydroxybenzyl and benzyl alcohols under anaerobic-light conditions. But *R. acidophila* strain 10050 did not grow on these three aromatic compounds. We first expected previously⁹⁾ that the ability to grow on some aromatic alcohols was one of the common characteristic features of *R. acidophila* to which Pfennig failed to refer.¹³⁾ However, two strains of *R. acidophila* showed clear differences in their behavior on the three benzyl

alcohols. Table I shows the growth on the three alcohols of several positive strains. They belong to *R. palustris* or *Rhodomicrobium* sp., presumably *Rhodomicrobium rubrum*, but none of them were preferentially active on these aromatic alcohols as *R. acidophila* M402 was.

The alcohols in the medium were consumed to some extent by these organisms during the incubation period of 170 hr, but the highest rate of consumption was observed with *R. acidophila* M402. Therefore, *R. acidophila*

TABLE III. EFFECT OF SUPPLEMENTATION OF ORGANIC ACIDS ON THE ENZYME PRODUCTION UNDER ANAEROBIC-LIGHT CONDITIONS

Culture were grown for 170 hr.

C-Source <i>p</i> -Hydroxybenzyl alcohol (3 mM) + organic acid (0.1%)	Bacterial growth ($A_{650\text{nm}}$)	Consumption of HBAL ^a in medium (%)	Crude extracts	
			Protein (mg)	<i>p</i> -HBAL dehydrogenase (units)
<i>p</i> -Hydroxybenzyl alcohol only	0.86	21.2	35.2	0.26
+ Acetate	1.86	32.8	60.8	0.32
+ Butyrate	1.98	26.6	62.1	0.29
+ Valerate	1.06	20.8	37.0	0.24
+ Malonate	0.87	20.4	36.0	0.20

^a HBAL = hydroxybenzyl alcohol.

M402 was selected and used throughout this experiment.

Cultures were grown on these aromatic alcohols as the major carbon source for 170 hr under anaerobic-light conditions. Dehydrogenase activities in the cell-free extracts were assayed on four benzyl alcohols, vanillyl alcohol, benzaldehyde and vanillin with NAD⁺ or PMS as the electron acceptor. The results are summarized in Table II. Anaerobically-grown cells did not produce any PMS-linked dehydrogenase from hydroxybenzyl or benzyl alcohols. *p*-Hydroxybenzyl alcohol-grown cells exhibited the activities of NAD⁺-linked dehydrogenase on *p*-hydroxybenzyl alcohol and benzyl alcohol. Benzyl alcohol-grown cells also produced the NAD⁺-linked dehydrogenases on benzyl alcohol, *p*-, *m*- and *o*-hydroxybenzyl alcohols and benzylaldehyde. *m*-Hydroxybenzyl alcohol was not effective as an inducer of these alcohol dehydrogenases. Vanillyl alcohol-grown cells however, produced the NAD⁺-vanillyl alcohol and/or vanillin dehydrogenases, and *p*-hydroxybenzyl alcohol and benzyl alcohol dehydrogenases. But PMS-linked dehydrogenase activities on vanillyl alcohol or *p*-hydroxybenzyl alcohol were detected in the same cells. Therefore, production of NAD⁺-aromatic alcohol dehydrogenases was specifically related to the cultural conditions. Anaerobic-light conditions were indispensable for this en-

FIG. 1. Time Course of Growth on *p*-Hydroxybenzyl Alcohol under Anaerobic-light Conditions.

The culture was performed with 2-liters of medium in a 2-liter Erlenmeyer flask. Samples (50 ml) were taken anaerobically every 24-hr. For each sample, the absorbance at 650 nm (●), *p*-hydroxybenzyl alcohol (○), *p*-hydroxybenzaldehyde (△) and *p*-hydroxybenzoic acid (□) were assayed. *p*-Hydroxybenzyl alcohol dehydrogenase activity (▲) was expressed as units/2 liters of medium.

zyme production. About 20% of the *p*-hydroxybenzyl alcohol in the medium was consumed after 190 hr cultivation under anaerobic-light conditions. Supplementation of acetate to the *p*-hydroxybenzyl alcohol medium enhanced the bacterial growth and the consumption of *p*-hydroxybenzyl alcohol. The

yield of dehydrogenase activity in the cells was also increased (Table III). But addition of other lower fatty acids such as butyrate or valerate also stimulated the bacterial growth, but was not effective for the enzyme production. No enzyme activity was found in the cells grown on acetate, butyrate, valerate, malonate or syringic acid.

Production of NAD⁺-p-hydroxybenzyl alcohol dehydrogenase

The time courses of the bacterial growth, consumption of *p*-hydroxybenzyl alcohol and accumulation of metabolic intermediates, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid, in the medium and production of dehydrogenase are illustrated in Fig. 1. NAD⁺-linked *p*-hydroxybenzyl alcohol dehydrogenase activity appeared after 70 hr cultivation, just after the initiation of bacterial proliferation and consumption of *p*-hydroxybenzyl alcohol. This enzyme activity reached the maximum after 120 hr, and then decreased rapidly. In the cultural fluid, *p*-hydroxybenzaldehyde accumulated, but it was later transformed to *p*-hydroxybenzoic acid which remained in the medium. Under these cultural conditions, PMS-linked *p*-hydroxybenzyl alcohol dehydrogenase and vanillyl alcohol dehydrogenase activities were not detected during the whole cultivation period.

Purification

Crude extract. The deep brownish red extract was used for further purification. To the crude extracts, protamine sulfate solution was added dropwise to 0.02 g protamine per g of protein. After standing for a further 20 min, precipitates were removed by centrifugation and discarded.

Ammonium sulfate fractionation. To the protamine supernatant, solid ammonium sulfate was added to 35% saturation and the resulting precipitates were removed by centrifugation. Reddish pigments remained mainly in the supernatant. More ammonium sulfate was added to bring the saturation to 85%. After stirring for 20 min, precipitates were collected

FIG. 2. Gel Filtration on Sepharose Cl 4B.

Symbols: ○, NAD⁺-*p*-hydroxybenzyl alcohol dehydrogenase; ▲, absorbance at 280 nm.

by centrifugation and dissolved in 20 ml of 50 mM Tris-HCl buffer, pH 7.5. The pH was adjusted to 7.5 with 0.2N NaOH during the fractionation.

Gel filtrations of Sepharose Cl-4B and Sephadex G-25. As active fractions were accompanied by the deep reddish pigments, bacteriochlorophyll and carotenoides, it was necessary to separate these pigments from the enzyme protein at an early stage of the purification. The exclusion gel filtration technique with Sepharose Cl-4B successfully separated these pigments from the enzyme activity as shown in Fig. 2. The ammonium sulfate fraction was passed through a column of Sepharose Cl-4B (2.7 × 37 cm) which was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.2M KCl. The column was washed with 500 ml of the same buffer at a flow rate of 20 ml per hr and 3 ml fractions were collected. Almost all of the pigments were eluted at the void volume. The enzyme activity appeared after elution of the pigment. Gel filtration on Sephadex G-25 was not suitable for this preparation, because pigments were retained in the gels. The active fractions were

TABLE IV. PURIFICATION OF *p*-HYDROXYBENZYL ALCOHOL DEHYDROGENASE OF *Rhodospseudomonas acidophila* M402

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein) × 1000	Recovery (%)	Relative purification
Crude extract	124	2,590	15.5	5.98	100	1
Protamine supernatant	135	1,420	13.3	9.37	85.8	1.57
Ammonium sulfate precipitate (35~85%)	27	820	9.5	11.59	61.3	1.94
Sephadex C1-4B eluate	42	350	7.8	22.29	50.3	3.73
Sephadex G-25 eluate	35	300	6.3	21.00	40.6	3.51
1st DEAE-cellulose	17	47	4.4	93.62	28.4	15.66
2nd DEAE-cellulose	16	11	1.7	154.55	10.97	25.84
1st Sephacryl S-300 eluate	4.2	6	0.9	150.00	5.80	25.08
2nd Sephacryl S-300 eluate	4.6	3	0.5	166.67	3.23	27.87

combined and condensed to about 10 ml through a Diaflo membrane under nitrogen gas pressure at 2°C for 30 min. The condensed solution was passed through the column of Sephadex G-25 (2.7 × 34.5 cm) which was equilibrated as above. Active fractions were pooled and condensed in the same manner prior to the next steps.

Column chromatographies on DEAE-cellulose. DEAE-cellulose was equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 M KCl. The condensed enzyme solution was applied to the column (2.7 × 35.5 cm) and the column was washed with 300 ml of the same buffer. Enzyme activity was recovered in the passed solution. The second DEAE-cellulose chromatography was carried out at pH 9.0. DEAE-cellulose was equilibrated with 50 mM glycine-NaOH buffer, pH 9.0, containing 0.1 M KCl. The first eluates were combined and condensed, and then applied to the column. After washing the column with 300 ml of buffer, enzyme activity was not found in the eluate. The enzyme was then eluted by the linear gradient technique with KCl at 0.3 M. Fractions (Nos. 162~188, 81 ml) were pooled and condensed to 16 ml.

Gel filtration on Sephacryl S-300. The enzyme solution was applied to a column of Sephacryl S-300 (1.5 × 75 cm) at pH 7.5 and proteins were eluted with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M KCl at a flow rate

FIG. 3. Molecular Weight by Gel Filtration on Sephacryl S-300.

Standard proteins used were: 1, cytochrome *c* (MW 12,500); 2, chymotrypsinogen *A* (MW 25,000); 3, ovalbumin (MW 45,000); 4, bovine serum albumin (MW 67,000); 5, aldolase (MW 158,000); ●, *p*-hydroxybenzyl alcohol dehydrogenase.

of 7 ml per hr. Fractions of 1 ml were collected. Active fractions were pooled and the procedure was repeated on the second Sephacryl S-300 column. The purification procedure is summarized in Table IV. The final preparation had a specific activity of 166.7 with 28-fold purification. This preparation was found to

FIG. 4. Isoelectric Point of *p*-Hydroxybenzyl Alcohol Dehydrogenase.

Symbols: ○, enzyme activity; ▲, absorbance at 280 nm; ●, pH.

FIG. 5. Effect of pH on Dehydrogenase Activity.

○, reduction of NAD^+ with *p*-hydroxybenzyl alcohol; ●, dehydrogenation of NADH with *p*-hydroxybenzaldehyde. Buffer were used: pH 5.0~7.0, 50 mM Citrate-phosphate; pH 7.5~8.5, 50 mM Tris-HCl; pH 9.0~10.5, 50 mM glycine.

FIG. 6. Effect of pH on Stability of *p*-Hydroxybenzyl Alcohol Dehydrogenase.

See legend to Fig. 5.

be homogeneous on disc electrophoresis on polyacrylamide gels with an R_m value of 0.20.

Molecular weight

The molecular weight of *p*-hydroxybenzyl alcohol dehydrogenase was estimated to be 27,000 dalton by the gel filtration method on Sephacryl S-300 (Fig. 3).

Isoelectric point. The isoelectric point of the enzyme was determined to be pH 7.4 by the ampholine electrofocusing method as shown in Fig. 4.

Properties

pH optimum. The effect of pH on the dehydrogenation reaction with NAD^+ was assayed in the pH range of 5 to 11 with *p*-hydroxybenzyl alcohol as substrate. The maximum activity was seen at pH 9.5 as shown in Fig. 5. That on the reverse reaction was also assayed with the reduction of *p*-hydroxybenzaldehyde with NADH. The maximum activity was observed at pH 6.5. While the dehydrogenation reaction was observed in the relatively wide range of pH 8 to 11, the reduction reaction occurred in the narrow

range of pH 6.5.

pH for stability. The effect of pH on enzyme stability was determined in the pH range of 5 to 10. The enzyme was stable at pH 7~10 (Fig. 6).

Substrate specificity. The enzyme showed affinity for a wide range of derivatives of *p*-substituted benzyl alcohols, aromatic alcohols and several aliphatic alcohols with NAD⁺ as cofactor. The results are summarized in Table V. The purified enzyme was active on *p*-hydroxybenzyl alcohol, but more active on vanillyl alcohol and cinnamyl alcohol. *p*-Substituted derivatives of benzyl alcohols also underwent dehydrogenation with NAD⁺, e.g. *p*-methoxy, *p*-nitro and *p*-chlorobenzyl alcohols. *m*-Derivatives of benzyl alcohols also underwent the dehydrogenation: *m*-methyl, *m*-methoxy, *m*-nitro and *m*-chlorobenzyl alcohols in that decreasing rate order. *o*-Derivatives of benzyl alcohols were inert for the enzyme. Benzyl alcohol was oxidized with 75% activity relative to the case of *p*-hydroxybenzyl alcohol. Other aromatic alcohols, such as phenoxy ethanol and phenyl ethanol, also underwent the dehydrogenation. All of these activities were detected at the same location on polyacrylamide gel (relative mobility=0.18~0.20). Each activity was detected on the gels with respective substrates. This evidence strongly suggested that a single enzyme catalyzed the dehydrogenation of these aromatic alcohols. The most striking feature of the substrate specificity of this enzyme is its wide specificity on aliphatic alcohols. *n*-Alcohols of C₄ to C₉ were actively oxidized by this enzyme. Among them, 1-octanol, 1-heptanol and 1-hexanol were the most active substrates. However, methanol and ethanol were completely inert for the enzyme. Moreover, 2-aliphatic alcohols were also inactive for this enzyme. A typical alcohol dehydrogenase (EC 1.1.1.1) was distributed in various organisms and active on primary alcohols other than methanol. The alcohol dehydrogenase induced by *p*-hydroxybenzyl alcohol is evidently different from this most common alcohol dehydrogenase. The follow-

TABLE V. SUBSTRATE SPECIFICITY

Substrate (3 mm)	Relative activity (%)	<i>R_m</i> ^a
<i>p</i> -Hydroxybenzyl alcohol	100	0.20
<i>m</i> -Hydroxybenzyl alcohol	92	0.21
<i>o</i> -Hydroxybenzyl alcohol	0	nd ^b
<i>p</i> -Methylbenzyl alcohol	93	0.19
<i>m</i> -Methylbenzyl alcohol	38	0.19
<i>o</i> -Methylbenzyl alcohol	0	nd
<i>p</i> -Methoxybenzyl alcohol	86	—
<i>m</i> -Methoxybenzyl alcohol	50	—
3,4-Dimethylbenzyl alcohol	0	—
<i>p</i> -Nitrobenzyl alcohol	80	0.19
<i>m</i> -Nitrobenzyl alcohol	53	0.18
<i>o</i> -Nitrobenzyl alcohol	0	—
<i>p</i> -Chlorobenzyl alcohol	88	0.19
<i>m</i> -Chlorobenzyl alcohol	55	0.18
<i>o</i> -Chlorobenzyl alcohol	0	—
Benzyl alcohol	75	0.21
Vanillyl alcohol	254	0.21
Cinnamyl alcohol	111	0.19
Coniferyl alcohol	0	—
Phenyl ethanol	33	0.20
Phenoxy ethanol	17	0.18
Methanol	0	—
Ethanol	0	—
1-Propanol	52	0.19
2-Propanol	0	—
1-Butanol	93	0.19
2-Methyl-1-propanol	38	0.20
2-Butanol	0	—
2-Methyl-2-propanol	0	—
1-Pentanol	97	0.20
3-Methyl-1-butanol	86	0.19
3-Methyl-2-butanol	0	—
2-Methyl-2-butanol	0	—
1-Hexanol ^c	111	—
1-Heptanol ^c	122	—
1-Octanol ^c	131	—
1-Nonanol ^c	98	—
1-Decanol ^c	11	—

^a Electrophoresis on 7.5% polyacrylamide gel at pH 9.3 was performed as in a previous paper.⁴⁾ The gels were incubated with the respective alcohols at 30°C for 20 min. The band of nitroblue tetrazolium reductase activity was located and recorded by the scanning technique. *R_m* (relative mobility) was expressed relative to bromphenol blue.

^b nd=not detectable.

^c Alcohols were dissolved in methanol. Methanol was inert for this enzyme, and did not inhibit or accelerate the activity on *p*-hydroxybenzyl alcohol. When these alcohols were dissolved in *N,N*-dimethylformamide, the activity was depressed to near one-tenth of the activity of the water-dissolved substrates.

TABLE VI. MICHAELIS CONSTANTS

Substrate	K_m (mM)
<i>p</i> -Hydroxybenzyl alcohol	8.91
<i>m</i> -Hydroxybenzyl alcohol	7.41
<i>p</i> -Methoxybenzyl alcohol	6.06
<i>m</i> -Methoxybenzyl alcohol	4.65
<i>p</i> -Methylbenzyl alcohol	9.10
<i>p</i> -Chlorobenzyl alcohol	9.71
Benzyl alcohol	5.64
Vanillyl alcohol	3.12
Cinnamyl alcohol	6.80
<i>p</i> -Hydroxybenzaldehyde ^a	2.17
1-Propanol	12.50
1-Butanol	9.22
1-Pentanol	10.60
1-Hexanol	7.40
1-Heptanol	6.51
1-Octanol	4.89
1-Nonanol	6.92

^a Reverse reaction with NADH.

ing compounds were examined but were found to be inert as substrates for the enzyme with NAD⁺: *o*-, *m*- and *p*-hydroxybenzaldehydes, benzaldehyde, vanillin, phenol, catechol, *o*-, *m*- and *p*-cresols, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dimethylphenols, cyclohexanol, 1,2-cyclohexane diol, formaldehyde, acetaldehyde and propionaldehyde. A comparison of the K_m values on these alcohols is presented in Table VI. Affinities of the enzyme toward various aromatic and aliphatic primary alcohols were about the same 2~10 mM. But the enzyme showed a little higher affinity for vanillyl alcohol, benzyl alcohol and cinnamyl alcohol. Substitution at the *p*-position of benzyl alcohol seemed not to cause significant changes in the affinity constants. Therefore, the enzyme should be a type of benzyl alcohol dehydrogenase having wide specificity.

Electron acceptor. NAD⁺ was required as an electron acceptor for the dehydrogenation on various alcohols, and NADP⁺ could not replace NAD⁺. The K_m value for NAD⁺ was found to be identical with two substrates: 0.58 and 0.60 mM with *p*-hydroxybenzyl alcohol and vanillyl alcohol, respectively (Table VII). These data provided the positive evidence that the two activities were catalyzed

TABLE VII. K_m FOR NAD⁺

Substrate (1 mM)	K_m (mM)
<i>p</i> -Hydroxybenzyl alcohol	0.58
Vanillyl alcohol	0.60

FIG. 7. Reaction Velocities on *p*-Hydroxybenzyl Alcohol and Vanillyl Alcohol.

Reactions were recorded at pH 9.5 with 2.5 mM vanillyl alcohol (○), *p*-hydroxybenzyl alcohol(△) and the two alcohols combined as substrate (■).

by a single enzyme.

Identity of *p*-hydroxybenzyl alcohol dehydrogenase and vanillyl alcohol dehydrogenase

The two activities on *p*-hydroxybenzyl alcohol and vanillyl alcohol were compared as to several aspects. Figure 7 shows that additivity of the two reaction velocities was not observed. The effects of addition of other alcohols on the two dehydrogenase activities were examined. As shown in Table VIII, addition of phenol was a little inhibitory for the two dehydrogenase activities. Among the alcohols tested, cresols (methyl phenol) clearly inhibited both activities to the same degree, 48.1 and 47.1% for *p*-hydroxybenzyl alcohol and vanillyl alcohol dehydrogenases with *m*-cresol, respectively.

All of these inhibitions with *m*- or *p*-cresols were competitive (Figs. 8 and 9). The K_i values for the two dehydrogenases were also identical

TABLE VIII. EFFECT OF ALCOHOLS AND PHENOLS ON AROMATIC ALCOHOL DEHYDROGENASE ACTIVITIES

Addition (2.5 mM)	Relative activities (%)	
	<i>p</i> -Hydroxybenzyl alcohol	Vanillyl alcohol
+ <i>p</i> -Hydroxybenzyl alcohol	—	105.5
+ <i>p</i> -Methylbenzyl alcohol	109.6	106.7
+ <i>p</i> -Chlorobenzyl alcohol	101.2	93.3
+ <i>p</i> -Methoxybenzyl alcohol	108.1	108.0
+ <i>o</i> -Cresol	104.5	100.0
+ <i>m</i> -Cresol	48.1	47.1
+ <i>p</i> -Cresol	59.6	56.8
+Phenol	86.5	76.7
+Catechol	97.7	102.3
+2,3-Dimethyl phenol	100.0	104.2
+2,4-Dimethyl phenol	93.2	96.1
+2,5-Dimethyl phenol	97.8	100.0
+3,4-Dimethyl phenol	86.4	95.5
+Cyclohexanol	93.2	103.1
+1,2-Cyclohexane diol	97.7	104.5
+Methanol	111.4	104.5
+Ethanol	102.1	101.1
+2-Propanol	111.3	102.7
+2-Butanol	104.5	104.2
+2-Methyl-2-propanol	102.2	108.8

FIG. 8. Inhibition of *p*-Hydroxybenzyl Alcohol Dehydrogenase with *m*- or *p*-Cresol.

A, control (without addition of a cresol); B, with 2.5 mM *m*-cresol; C, with 2.5 mM *p*-cresol.

with *m*-cresol or *p*-cresol (Table IX). This evidence also supported that the two dehydrogenase activities were due to a single enzyme.

FIG. 9. Inhibition of Vanillyl Alcohol Dehydrogenase with *m*- or *p*-Cresol.

A, control (without addition of a cresol); B, with 2.5 mM *m*-cresol; C, with 2.5 mM *p*-cresol.

TABLE IX. INHIBITION CONSTANTS OF CRESOLS

Inhibitor	<i>p</i> -Hydroxybenzyl alcohol <i>K_i</i> (mM)	Vanillyl alcohol
+ <i>m</i> -Cresol	1.25	1.35
+ <i>p</i> -Cresol	2.59	2.91

DISCUSSION

We have demonstrated in this study a new type of NAD⁺-linked alcohol dehydrogenase from cells of *Rhodopseudomonas acidophila* M402 grown anaerobically on *p*-hydroxybenzyl alcohol. The substrate specificity and affinity led to the conclusion that this enzyme was a kind of benzyl alcohol dehydrogenase which showed the highest affinity for vanillyl alcohol. Some properties of this enzyme resembled those of the benzyl alcohol dehydrogenase from *Pseudomonas* sp.¹⁴⁾ This enzyme was active on benzyl alcohol and salicyl alcohol (*o*-hydroxybenzyl alcohol) to the same degree, and less active on phenyl ethanol(25), 1-hexanol(19), 1-heptanol(6) and 1-octanol(2). The relative activity with benzyl alcohol was tentatively taken as 100 and each activity is shown in parenthesis. This enzyme was also inactive on methanol, ethanol, 1-propanol, isopropanol, 1-butanol, isobutanol and cyclohexanol. Judging from these data, the two benzyl alcohol dehydrogenases showed common properties, but were different in

substrate specificity and enzyme stability. The *Pseudomonas* enzyme was unstable, it only had a half-life of a few hours under conventional conditions.¹⁴⁾ The *R. acidophila* M402 enzyme was stable. The enzyme activity was always recovered after over one month at 0~5°C. Another dehydrogenase acting on hydroxybenzyl alcohol was reported in *Penicillium urticae*.¹⁵⁾ This enzyme was active on *m*-hydroxybenzyl alcohol with NADP⁺. *p*-Aminobenzyl alcohol dehydrogenase from *Mycobacterium tuberculosis* was called on aryl-alcohol dehydrogenase (EC 1.1.1.91).¹⁶⁾ This enzyme catalyzed the interconversion of *p*-aminobenzyl alcohol and *p*-aminobenzaldehyde as well as benzyl alcohol and benzaldehyde. The enzyme was incapable of oxidizing either methanol or ethanol at pH 6.8, and less rapidly cyclohexane methanol. At pH 7.8, butanol and phenyl ethanol were oxidized at approximately 30% the rate for *p*-aminobenzyl alcohol (pH 6.8). The substrate specificity of the enzyme was not further studied in detail. The enzyme utilized either NAD⁺ or NADP⁺. Three different aromatic alcohol dehydrogenases were partially purified from *Pseudomonas putida* N.C.I.B. 9869 grown on either 3,5-xylene or *p*-cresol.¹¹⁾ They were different in molecular weight: 145,000 (3,5-xylene-grown, activity 1), 122,000 (activity 2) and 75,000 (*p*-cresol-grown), but they were active on *m*- or *p*-hydroxybenzyl alcohols, *m*- or *p*-methylbenzyl alcohols and benzyl alcohol, but showed different ratios. The metabolic pathway of coniferyl alcohol was proposed with *Nocardia* sp.¹⁷⁾ Participation of a specific dehydrogenase in the first step of conversion of coniferyl alcohol to coniferyl aldehyde was expected, but not mentioned. Comparing the substrate specificity of these aromatic alcohol dehydrogenases,

the enzyme from *R. acidophila* M402 might be regarded as a NAD⁺-aryl alcohol dehydrogenase (EC 1.1.1.90). Differences between the two enzymes were observed as to several aspects. Therefore, we adopt the name of a new NAD⁺-*p*-hydroxybenzyl alcohol dehydrogenase for this enzyme.

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