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METABOLITES OF *p*-AMINOBENZOIC ACID

V. ISOLATION AND PROPERTIES OF *p*-AMINOBENZYL ALCOHOL DEHYDROGENASE

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SUMMARY

An aryl alcohol dehydrogenase (aryl alcohol: $NADP^+$oxidoreductase, EC 1.1.1.91), that catalyzes the interconversion of benzyl alcohol and benzaldehyde as well as the oxidation of *p*-aminobenzyl alcohol has been isolated from *Mycobacterium tuberculosis* cells (American Type Culture Collection No. 607) grown in the presence of *p*-aminobenzoic acid. The partially purified enzyme shows a major peak with a minor contaminating component upon ultracentrifugation.

The enzyme shows a broad substrate specificity and is sensitive to the buffer composition. The enzyme is inhibited by *p*-chloromercuribenzoate and 1,10-*o*-phenanthroline.

The apparent K_m values and the calculation of the approximate equilibrium constant are also presented.

INTRODUCTION

Previous studies by Sloane and co-workers[1–6] on the metabolism of *p*-aminobenzoic acid by acid-fast bacteria revealed the reduction of *p*-aminobenzoic acid to *p*-aminobenzyl alcohol. Since it appeared probable that the aldehyde was an intermediate in this reaction, a study was undertaken to (a) isolate and purify the enzyme that is capable of oxidizing *p*-aminobenzyl alcohol and (b) determine the properties of this enzyme.

It is the purpose of this paper to present data on the characteristics of this enzyme and to describe a simple method for its partial purification.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

METHODS

Preparation of induced cells

Cell-free extracts were prepared from *Mycobacterium tuberculosis* (American Type Culture Collection No. 607) that were grown in the basal medium previously described containing 1 g *p*-aminobenzoic acid per liter[1]. The cells were grown under conditions of forced aeration in 5-l quantities in a 9-l bottle at 33–37 °C for approximately 96 h. The inoculum consisted of a 50–100 ml of culture grown in shaker flasks for 48–72 h. After the growth period the metabolism of *p*-aminobenzoic acid was verified by the presence of *p*-aminophenol by the use of the indophenol blue test[6]. The cells were collected by centrifugation and washed with cold water; excess moisture was removed by squeezing cells in gauze. 80 g (semi-dried cells) were suspended in cold 0.05 M Tris–HCl buffer (pH 7.5) and the volume was adjusted to 100 ml; the cell suspension was sonicated in a 10-kcycle Raytheon sonic oscillator for a total of 2 min (8–15-s energy bursts; the temperature was maintained at 7–15 °C); the sonicated cells were centrifuged at 10 000 × *g* at 0–2 °C; the debris was washed with the Tris–HCl buffer (pH 7.5, 0.05 M) and the volume was adjusted to 100 ml.

p-Aminobenzyl alcohol dehydrogenase assay

The assay of the aryl alcohol dehydrogenase (aryl alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.91) was determined by the rate of $NADP^+$ reduction at 340 nm (25 °C) with continuous scanning utilizing a Cary Model 11 spectrophotometer equipped with an expanded scale slide wire (0–0.10). The reaction mixture contained imidazole buffer (0.05 M, pH 6.8), 400 μg NADP (Sigma) and 1 mg (*p*-aminobenzyl alcohol)$_2$oxalate and the enzyme in a total volume of 3.0 ml. One unit of activity was defined as 1 nmole of NADPH formed per min from *p*-aminobenzyl alcohol in imidazole buffer (0.05 M) at pH 6.8 at 25 °C. The concentration of NADPH formed was calculated utilizing the ε of $6.22 \cdot 10^3$ for the NADPH (ref. 7). When benzyl alcohol was the reactant, it was added in a concentration of 2 mg per 3.0 ml in the appropriate buffer as indicated in the tables. Specific activity was expressed in units/μg of protein. The protein concentration was determined by the method of Lowry *et al.*[8] utilizing crystalline bovine serum albumin (Sigma) as the standard. The reverse reaction, benzaldehyde reduction was measured at 340 nm utilizing NADPH (Sigma) as the hydrogen donor in a concentration of 40 μg in 3.0 ml. The benzaldehyde concentration was 100 μg in 3.0 ml. The *p*-chloromercuribenzoate (PCMB) was obtained from Mann Chemical Co., New York, New York. A 10^{-2} M solution in 0.05 M Tris, pH 7.5, was prepared by the addition of minimal 2 M NaOH to effect solution; 0.1 ml of 10^{-2} M PCMB added to 2.0 ml of imidazole buffer (0.05 M), pH 6.8, raised the pH to 7.1; whereas, 0.05 ml of 10^{-2} M PCMB added to 2.95 ml of imidazole buffer did not affect the pH (final concentration of $1.67 \cdot 10^{-4}$ M). The 1,10-*o*-phenanthroline was prepared as a 10^{-2} M solution in water.

An approximation of the equilibrium constant

To 0.664 μmole of NADPH and 0.693 μmole of benzaldehyde were added 0.5 ml enzyme (170 μg protein of 0.5–0.75 saturated $(NH_4)_2SO_4$ fraction of the NADPH eluate) in 0.05 M imidazole buffer, pH 6.8, in a total volume of 3.0 ml.

The reaction was performed at 25 °C and the rate of reduction of benzaldehyde was followed with the Cary Model 11 spectrophotometer at 340 nm. Equilibrium was reached after 30 min. The concentration of $NADP^+$ formed was calculated from the decrease in absorbance of NADPH. At equilibrium the absorbance was 0.11, whereas, at t_0 the absorbance was 1.42 at 340 nm.

RESULTS AND DISCUSSION

Purification of the p-aminobenzyl alcohol dehydrogenase

The sonicate contained 8.8 mg of protein per ml with a specific activity of 0.015 units/μg of protein. At 0–2 °C, 6.4 ml of 2% protamine sulfate solution (adjusted to pH 7.0) were added with stirring to 50 ml of the soluble fraction (equivalent to 40 g of cells) and the mixture was allowed to sit in ice for 10 min before the protamine nucleate was recovered by centrifugation. The supernate was devoid of enzymic activity. The precipitate was washed 3 times with 5-ml volumes of cold 0.05 M Tris–HCl buffer (pH 8.0). The washed precipitate was suspended in 6.0 ml of the Tris–HCl buffer (pH 8.0) and 4.0 ml of $NADP^+$ (16 mg) at pH 7 were added; the mixture was allowed to sit in ice with occasional stirring for 1 h before centrifugation. The precipitate was washed with a small volume of buffer (pH 8.0); the washings were added to the eluate. The $NADP^+$ eluate showed a specific activity of 0.036 unit/μg of protein with a 31.57% recovery of activity. The $NADP^+$ eluate was further purified by $(NH_4)_2SO_4$ precipitation; to 10 ml of the eluate at 0–2 °C (equivalent to 40 g of 607 cells) were slowly added 3 vol. of cold saturated $(NH_4)_2SO_4$ solution (adjusted to approximately pH 8 with NH_4OH using pHydrion paper (Micro-Essential Laboratory, Brooklyn, N.Y.). The 0.75-saturated $(NH_4)_2SO_4$ precipitate was collected by centrifugation. The precipitate was dissolved in 4.5 ml cold 0.05 M Tris–HCl buffer (pH 7.5) and 4.5 ml of the cold-saturated $(NH_4)_2SO_4$ solution were slowly added. The 0.5-saturated $(NH_4)_2SO_4$ precipitate was removed by centrifugation and to the $(NH_4)_2SO_4$ solution were slowly added 9.0 ml of 1-saturated $(NH_4)_2SO_4$ solution (pH 8) and set at 2–4 °C for 18 h. The 0.5–0.75-saturated $(NH_4)_2SO_4$ fraction showed schlierin upon gentle rotation of the flask. This fraction had a specific activity of 0.152 unit/μg of protein with an 18% overall recovery of activity. This represented a 10-fold increase in activity based on the sonicate. The results are summarized in Table I. The ultracentrifuge pattern of the 0.5–0.75-saturated $(NH_4)_2SO_4$ fraction showed a single major component with a small amount of a faster moving component (Fig. 1).

Suhara *et al.*[9] reported a 116-fold purification of benzyl alcohol dehydrogenase from *Pseudomonas* sp. to homogeneity, with a 10% overall yield. More recently, Forrester and Gaucher[10] purified the enzyme *m*-hydroxybenzyl alcohol dehydrogenase from *Penicillium urticae* 6.55-fold with a 15% overall recovery of activity.

Effect of pH and buffer system on enzymic activity

The results of a study of the effect of both the pH and the buffer system on the activity of the enzyme are shown in Fig. 2. It can be readily seen that both the pH and the buffer have a profound effect on the activity of enzyme with *p*-aminobenzyl alcohol as the substrate. In imidazole buffer the enzyme shows a high activity between pH 6.2–7.2; the pH optimum is 6.8. In phosphate buffer the optimal activity

TABLE I

PURIFICATION OF *p*-AMINOBENZYL ALCOHOL DEHYDROGENASE

Fraction	*Total protein (mg)*	*Total units**	*Specific activity (units/μg protein) × 10³*	*Activity recovered** (%)*
Sonicate	342	5210	15.2	100
$NADP^+$ eluate	45.6	1640	36.0	31.5
$(NH_4)_2SO_4$ fraction (0.5–0.75 saturated)	6.27	953	152.0	18.3

* 1 unit = 1 nmole of NADPH formed per min from *p*-aminobenzyl alcohol at pH 6.8 in imidazole buffer (0.05 M) at 25 °C, as described in the text.

** Based on the soluble protein in the sonicate.

is between pH 7.0–8.0 but the extent of activity of the enzyme in phosphate buffer is only one-half that shown in imidazole buffer. In the Tris buffer optimal activity is shown between pH 7.9 and 8.5, the enzyme is about 40% as active in this buffer as

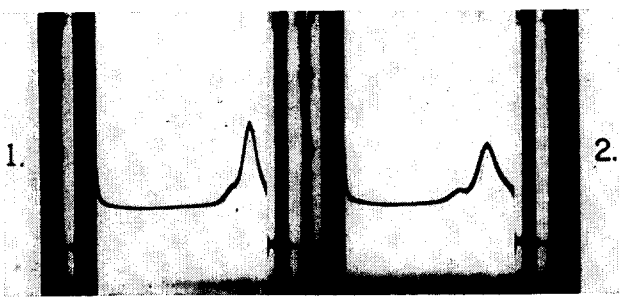

Fig. 1. Ultracentrifuge pattern of partially purified enzyme. The protein concentration was 8.4 mg/ml in 0.05 M phosphate, pH 7.5, with 0.2 M NaCl. Centrifugation was performed at 25 °C. Picture 1 was obtained 26 min after reaching speed of 59 780 rev./min. Picture 2 was obtained 42 min after reaching speed.

in the imidazole buffer. The results in Fig. 3 show that the benzyl alcohol dehydrogenase activity of the enzyme is a function of both the pH and the buffer but the degree of the influence of these variables is not as great as with *p*-aminobenzyl alcohol as the substrate. The optimal pH in imidazole (0.05 M) is at 8.0. The activity in Tris is approximately 75% of the activity found in imidazole or phosphate to buffer.

Specificity of aryl hydroxymethyl dehydrogenase

The results in Fig. 4 show that both benzyl alcohol and cyclohexane methanol are oxidized less rapidly than *p*-aminobenzyl alcohol; the rate of oxidation of cyclohexane methanol was about one-half the rate of that of benzyl alcohol. The enzyme utilized either NAD^+ or $NADP^+$ in these oxidations. The enzyme was incapable of oxidizing either methanol or ethanol. At pH 7.8 in imidazole buffer (0.05 M) both butanol and phenylethanol are oxidized at approximately 0.30 the rate of *p*-aminobenzyl alcohol (pH 6.8). The enzyme was incapable of reducing either *p*-aminobenzoic acid or benzoic acid in the presence of NADPH. The reverse reaction, the reduction

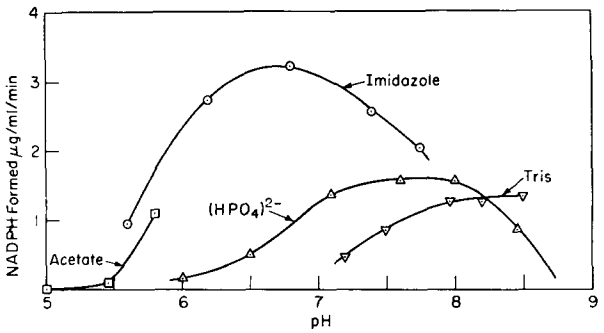

Fig. 2. Effect of pH and buffer upon the activity of *p*-aminobenzyl alcohol dehydrogenase. The conditions for performing the assay are described in the text. The enzyme preparation was the sonicate at a concentration of 225 μg protein per ml.

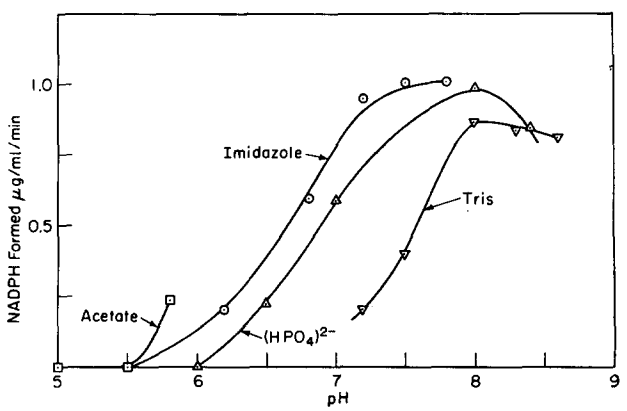

Fig. 3. Effect of pH and buffer upon benzyl alcohol oxidation by the enzyme. The conditions for performing the assay are described in the text. The sonicate was utilized at a concentration of 125 μg protein per ml.

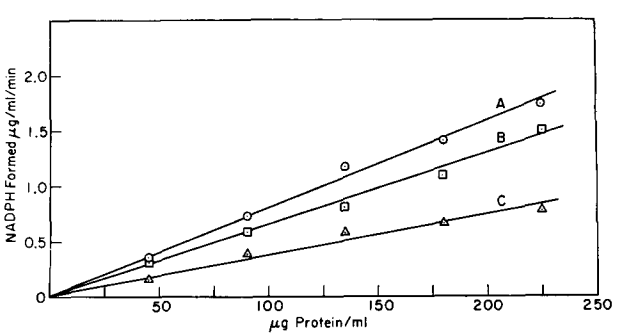

Fig. 4. Effect of protein concentration and substrate upon the oxidation of alcohols. The conditions for performing the assay are described in the text. The sonicate was utilized to determine enzymic activity. A, *p*-aminobenzyl alcohol (333 μg/ml) in imidazole buffer (0.05 M, pH 6.8). B, benzyl alcohol (666 μg/ml) at pH 7.8 in the imidazole buffer. C, cyclohexane methanol (666 μg/ml) at pH 7.8 in imidazole buffer.

of benzaldehyde to benzyl alcohol is clearly favored; the aldehyde is reduced at approximately three times the rate of alcohol oxidation (Table II). Forrester and Gaucher[10] reported that reduction of *m*-hydroxybenzaldehyde to the alcohol is approximately six times the rate of alcohol oxidation.

TABLE II

EFFECT OF *p*-CHLOROMERCURIBENZOATE AND 1,10-*o*-PHENANTHROLINE ON THE ACTIVITY OF THE DEHYDROGENASE

The enzyme was the 0.5–0.75-saturated $(NH_4)_2SO_4$ fraction (150 μg/ml) stored at 4 °C for 3 months. The reactions were run in 0.05 M imidazole buffer (pH 6.8). The conditions were those described in the text.

Substrate	*NADP⁺ or NADPH reacted per 3 ml per min at 25 °C (ng)*
1 Benzaldehyde (10.5 μg)	4300
2 *p*-Amino benzyl alcohol (200 μg)	3950
3 Benzyl alcohol (1 mg)	1440
4 1 + PCMB ($3.33 \cdot 10^{-4}$ M)	890
5 1 + PCMB ($1.67 \cdot 10^{-4}$ M)	1260
6 2 + PCMB ($1.67 \cdot 10^{-4}$ M)	0
7 2 + PCMB ($6.60 \cdot 10^{-5}$ M)	0
8 3 + PCMB ($6.60 \cdot 10^{-5}$ M)	0
9 1 + 1,10-*o*-phenanthroline ($3.3 \cdot 10^{-4}$ M)	1190
10 1 + 1,10-*o*-phenanthroline ($1.67 \cdot 10^{-4}$ M)	1800
11 2 + 1,10-*o*-phenanthroline ($3.3 \cdot 10^{-4}$ M)	400
12 3 + 1,10-*o*-phenanthroline ($1.67 \cdot 10^{-4}$ M)	80

Suhara *et al.*[9] and Katagiri *et al.*[11] demonstrated that the aryl alcohol dehydrogenase isolated from *Pseudomonas* sp. utilized either NAD⁺ or NADH specifically; whereas, Forrester and Gaucher[10] showed that the dehydrogenase from *Penicillium urticae* utilized NADPH more effectively than NADH.

K_m determinations. The apparent K_m values were determined by Lineweaver–Burk plots. Plots of initial rates *vs* substrate concentrations were typical hyperbolic curves indicating classical Michaelis–Menten kinetics. The following K_m values were determined: NADP⁺ (with benzyl alcohol (666 μg/ml) as substrate) $5.7 \cdot 10^{-5}$ M in Tris, 0.05 M at pH 8.0; NADP⁺ (with *p*-aminobenzyl alcohol (333 μg/ml) as substrate) $4.0 \cdot 10^{-5}$ M in imidazole, 0.05 M at pH 6.8; *p*-aminobenzyl alcohol $1.8 \cdot 10^{-2}$ M in imidazole buffer, 0.05 M at pH 6.8; benzyl alcohol $1.1 \cdot 10^{-2}$ M in Tris, 0.05 M at pH 8.0. The concentration of NADP⁺ in the latter two experiments was 133 μg/ml. The enzyme affinity for these substrates is approximately 0.03 of that shown by benzyl alcohol with benzyl alcohol dehydrogenase as described by Suhara *et al.*[9].

Effect of inhibitors on the dehydrogenase

The results recorded in Table II show that the dehydrogenase reaction involving the oxidation of the alcohol to the aldehyde is sensitive to PCMB in the presence of NADP⁺, but the reduction of the aldehyde in the presence of NADPH is partially protected from the inhibitory action of the sulfhydryl-reacting reagent.

Aryl alcohol oxidation (benzyl alcohol) is completely inhibited by $6.60 \cdot 10^{-5}$ M

PCMB whereas aldehyde reduction in the presence of NADPH was inhibited 70%, by $1.67 \cdot 10^{-4}$ M PCMB (Table II).

Furthermore, a similar pattern of enzymic protection from inhibition by 1,10-*o*-phenanthroline is shown in the presence of NADPH in the enzymic reaction catalyzed by the enzyme. The oxidation of *p*-aminobenzyl alcohol is inhibited to the extent of 90% by phenanthroline ($3.30 \cdot 10^{-4}$ M) and the oxidation of benzyl alcohol is inhibited 95% (at $1.67 \cdot 10^{-4}$ M), whereas, in the presence of NADPH the reduction of benzaldehyde is inhibited to the extent of 58% in the presence of this inhibitor at $1.67 \cdot 10^{-4}$ M concentration.

The cofactor NADPH has been shown by Forrester and Gaucher[10] to protect the enzyme (*m*-hydroxybenzyl alcohol dehydrogenase) against inactivation by iodoacetate and diethylpyrocarbonate. Subato and Kaplan[12] and Auricchio and Bruni[13] reported that NADH protects the essential sulfhydryl groups of lactic dehydrogenase and yeast alcohol dehydrogenase from inactivation by PCMB and iodoacetate.

The benzyl alcohol dehydrogenase[11] obtained from *Pseudomonas* sp. was not inhibited by phenanthroline ($3 \cdot 10^{-3}$ M), but this enzyme was inhibited by sulfhydryl-reacting reagents PCMB and *N*-ethylmaleimide[11].

An approximation of the equilibrium constant

At equilibrium the concentration of benzyl alcohol was assumed to be equal to the concentration of NADPH utilized in the reaction. The concentration of benzaldehyde was calculated to be the difference between the benzaldehyde at t_0 and the benzyl alcohol concentration at equilibrium. The apparent equilibrium constant for the dehydrogenase reaction could be calculated as: K'_{app} = [aldehyde] [NADPH]/[alcohol] [NADP] = 0.071 (pH 6.8, imidazole buffer (0.05 M), at 25 °C). The reaction favors the reduction of aldehyde to the alcohol. The standard free energy change $\Delta G^{0'} = +1.58$ kcal/mole. These values are in the same range as the values for *m*-benzyl alcohol dehydrogenase isolated from *Penicillium urticae*; Forrester and Gaucher[10] reported values of 0.18 for K'_{app} at 30 °C and +1.04 kcal/mole for $\Delta G^{0'}$.

The properties of the enzyme, aryl alcohol dehydrogenase from three different sources have now been reported. The most striking similarity between these enzymes is the somewhat non-specific substrate specifically exhibited. The enzymes differ in other respects as noted above.

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