Substrate Specificity of Formaldehyde Dehydrogenase from *Pseudomonas putida*

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The substrate specificity of formaldehyde dehydrogenase purified from a cell-free extract of *Pseudomonas putida* C-83 was reinvestigated with a series of aldehyde and alcohol substrates. The activities toward formaldehyde and n-butanol were almost parallel throughout the procedures of enzyme purification and pH-stability experiments, suggesting that the same enzyme protein shows dehydrogenase activities for both aldehyde and alcohol substrates. Formaldehyde was the best substrate and the enzyme showed reduced activity with increasing length of alkyl groups of aldehydes, no activity being detected for n-butyraldehyde. Among the alcohol substrates, n-pentanol was the best substrate, and no activity was observed for methanol or ethanol. The enzyme activity for alcohols increased with increasing length of alkyl groups until n-pentanol and then decreased gradually. The dehydrogenase activity toward formaldehyde was inhibited competitively by other aldehydes but noncompetitively by n-butanol and n-hexanol. On the other hand, the type of inhibition by aldehydes for n-butanol dehydrogenation varied depending upon the length of alkyl groups of aldehydes used.

We have previously reported the purification and some properties of four enzymes involved in catabolism of creatinine by *Pseudomonas putida* C-83, creatinine amidohydrolase, creatine amidinohydrolase, sarcosine dehydrogenase and formaldehyde dehydrogenase.1–6 The last enzyme was NAD+ dependent but did not require glutathione as a co-factor and did not catalyze the reverse reaction (hydrogenation of formate to formaldehyde), different to the usual formaldehyde dehydrogenases hitherto reported.7,8 Throughout the purification of the enzyme, it was found that the active fractions for formaldehyde were always accompanied by the dehydrogenase activity toward n-butanol, there being an almost constant ratio of the activities for both substrates.

In order to clarify whether the two activities are chromatographically separable or not, the enzyme was again highly purified by the previous method with modifications, and its substrate specificity was reexamined in detail by using a series of aldehyde and alcohol substrates. The present paper deals with the substrate specificity of the formaldehyde dehydrogenase purified to homogeneity from a cell-free extract of *Pseudomonas putida* C-83.

**MATERIALS AND METHODS**

*Materials.* The following reagents were purchased from Nakarai Kagaku Co., Kyoto: Phenazine methosulfate (PMS), nitro blue tetrazolium (NBT) and 3-methyl-2-benzothiazolinone (MBT). NAD+, NADH and NADP+ were from Kojin Kagaku Co., Tokyo. Aldehydes and alcohols were from E. Merck, Germany. Sephadexs, DEAE-Sephadex and phenyl Sepharose CL-4B were products of Pharmacia Fine Chemicals, Sweden. Hydroxyapatite was prepared following the method described by Bernardi.9

*Enzyme activity assay.* (A) *Diformazan formation:* During the purification of formaldehyde dehydrogenase,

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Abbreviations: MBTH, 3-methyl-2-benzothiazolinone hydrazone; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate.
the enzyme activity was assayed by the formation of diformazan by following the increase in absorbance at 570 nm after coupling the NAD⁺ reduction with PMS and NBT. To 0.5 ml of 4.8 mm HCHO in 50 mm Tris-HCl buffer, pH 7.8, containing 0.5% Triton X-100, were added 0.1 ml of 12 mm NAD⁺ and 0.1 ml of a mixture of 0.05% each of PMS and NBT in water. The reaction was started by adding 0.5 ml of the enzyme to the above solution at 37°C. After incubation for 15 min, the reaction was stopped by adding 3 ml of 0.3 N HCl, and the absorbance at 570 nm was measured with a Hitachi 100-10 spectrophotometer. The activity in units was calculated with the following equation, for which one unit was defined as the amount of enzyme that formed one-half μmol of diformazan per min under the conditions.

Units/ml = ΔE₅₇₀ × 0.028 × dilution factor

The dehydrogenase activity on n-butanol was also assayed by the method described above, except that the final concentration of n-butanol was 0.1 m. Protein concentration was estimated spectrophotometrically assuming that A₄₁₀ at 280 nm is 10, and the specific activity was expressed as activity units per mg protein.

(B) NADH formation: For the purified enzyme, the dehydrogenase activity was assayed in terms of the increase in absorbance at 340 nm due to the formation of NADH using a Shimadzu UV-300 spectrophotometer equipped with a thermostatted cell compartment. One ml of 2.4 mm formaldehyde or 0.12 m n-butanol in 60 mm sodium carbonate-bicarbonate buffer of the respective optimal pH was incubated at 37°C with 0.1 ml each of the enzyme and 12 mm NAD⁺. The change in optical density at 340 nm was followed for several min and the initial rate was expressed as ΔE₃₄₀ per min.

Kinetic studies. The enzyme reaction was carried out by method B, except that various concentrations of aldehydes and alcohols and a fixed, saturated concentration of NAD⁺ were used. The apparent Km and Vₘₐₓ values were estimated according to the best fitting plots method using a Commodore microcomputer 3032. The apparent Km value for NAD⁺ was also estimated by the same method, except that fixed and saturated concentrations of aldehydes and alcohols were used. Ki values of various aldehydes and alcohols were calculated from Lineweaver-Burk plots.

Stoichiometry of the formation of formic acid from formaldehyde and of NADH from NAD⁺ was confirmed as described previously. The formation of butyraldehyde from n-butanol was also confirmed by the method of Sawicki et al. by using 3-methyl-2-benzothiazolinone hydrazone (MBTH method).

Purification of enzyme. Pseudomonas putida C-83 was cultured and disrupted in the same manner as reported previously, and the cell-free extract, 11 liters, was used for the purification of the enzyme. All procedures were carried out at 4°C. A part of the cell-free extract, 450 ml, was fractionated by precipitation with 35 ~ 60% saturation with ammonium sulfate. The resulting precipitate was collected by centrifugation, dissolved in 40 ml of 10 mm Tris-HCl buffer, pH 7.2, and then passed through a column (3 x 90 cm) of Sephadex G-25 previously equilibrated with the above buffer to remove ammonium sulfate. The active fractions were combined, applied to a column (3 x 30 cm) of DEAE-Sephadex A-50 equilibrated with the above buffer, and after the column was washed with the buffer, the adsorbed enzyme was eluted with an increasing linear concentration gradient of NaCl to 0.3 M in a total volume of 1.2 liters. Active fractions were combined and subjected, under 10% saturation with ammonium sulfate, to hydrophobic chromatography on a phenyl-Sepharose CL-4B column (2.4 x 30 cm) previously equilibrated with the above buffer to remove 10% saturation with ammonium sulfate. The adsorbed enzyme was eluted with a decreasing linear gradient of ammonium sulfate (10% saturation to 0%) in 250 ml each of the same buffer. After concentration by ultrafiltration with an Amicon PM-10, the enzyme solution was subjected to gel filtration on a column (2 x 110 cm) of Sephadex G-150 equilibrated with 10 mM phosphate buffer, pH 6.8. The active fractions were combined and applied to a column (1.5 x 15 cm) of hydroxyapatite equilibrated with the same buffer, and the adsorbed enzyme was eluted with an increasing linear gradient of phosphate to 0.5 M in a total volume of 300 ml. Active fractions of constant specific activity (11.2 ~ 11.4) were combined, concentrated by ultrafiltration and then dialyzed against 10 mM phosphate buffer, pH 6.8. The remaining part of the cell-free extract was also subjected to the same procedures and the final preparation was stored at −20°C until use.

RESULTS AND DISCUSSION

Purification and some properties of the enzyme

Table I summarizes the purification of formaldehyde dehydrogenase of Pseudomonas putida C-83, and the chromatogram on hydroxyapatite is shown in Fig. 1. As shown in Table I, the ratio of dehydrogenase activities for formaldehyde and n-butanol was approximately constant throughout the purification procedures, and the elution profile of the formaldehyde dehydrogenase activity on a hydroxyapatite column completely coincided with that of the activity for n-buta-
Table I. Purification of Formaldehyde Dehydrogenase from Pseudomonas putida C-83

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity*</th>
<th>S.A.</th>
<th>Activity ratio^b</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free extract</td>
<td>450</td>
<td>16,980</td>
<td>2560</td>
<td>0.15</td>
<td>0.25</td>
<td>100</td>
</tr>
<tr>
<td>Salted out with (NH₄)₂SO₄</td>
<td>52</td>
<td>2,600</td>
<td>1742</td>
<td>0.67</td>
<td>0.24</td>
<td>68</td>
</tr>
<tr>
<td>Desalted with Sephadex G-25</td>
<td>130</td>
<td>2,113</td>
<td>1463</td>
<td>0.69</td>
<td>0.22</td>
<td>57</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50</td>
<td>150</td>
<td>377</td>
<td>942</td>
<td>2.50</td>
<td>0.24</td>
<td>37</td>
</tr>
<tr>
<td>Phenyl Sepharose CL-4B</td>
<td>200</td>
<td>120</td>
<td>589</td>
<td>4.91</td>
<td>0.23</td>
<td>23</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>95</td>
<td>85</td>
<td>510</td>
<td>11.3</td>
<td>0.23</td>
<td>20</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>190</td>
<td>29</td>
<td>330</td>
<td>11.3</td>
<td>0.23</td>
<td>13</td>
</tr>
</tbody>
</table>

*Activity was assayed in terms of diformazan formation.

Activity units.

*HCHO = n-Butanol at pH 7.8.

S.A.: Specific activity of formaldehyde dehydrogenase.

Fig. 1. Chromatogram of the Enzyme on a Hydroxyapatite Column.

About 250 units of activity for formaldehyde were applied to a column (1.5 x 15 cm) of hydroxyapatite equilibrated with 10 mM phosphate buffer, pH 6.8. The adsorbed enzyme was eluted with a linear gradient formed from 150 ml each of 10 mM and 500 mM phosphate buffer, pH 6.8. The enzyme activities were assayed by diformazan formation.

O, activity for formaldehyde; •, activity for n-butanol.

The final preparation was electrophoretically homogeneous as judged by disc gel and SDS gel electrophoreses and both activities were found in the region of the protein peak (Fig. 1).

The purified enzyme, 22 μg, in 0.5 ml of 0.1 M buffers of various pHs was incubated at 30°C for 60 min. The residual activities were assayed using 0.1 ml aliquots of the incubation mixtures by method B at respective optimal pHs. Buffers used were: acetate (pH 4.5), phosphate (pH 6.2 ~ 7.2), Na₂CO₃–NaHCO₃ (pH 9.0 ~ 10.2) and NaOH–KCl (pH 11.5 ~ 12.3).

O, activity for formaldehyde; •, activity for n-butanol.

Fig. 2. Effect of pH on Stabilities of Dehydrogenase Activities for Formaldehyde and n-Butanol.

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O, activity for formaldehyde; •, activity for n-butanol.

(data not shown). As shown in Fig. 2, the pH-stability profile was almost the same for both dehydrogenase activities. These results led us to conclude that the same enzyme protein exhibits both dehydrogenase activities.

However, the optimum pHs for the activities on aldehydes and alcohols are clearly distinguishable from each other. The enzyme was
Fig. 3. Effect of pH on Dehydrogenase Activities for Formaldehyde and n-Butanol.

Table II. Kinetic Parameters of Formaldehyde Dehydrogenase for Various Aldehydes and Alcohols

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ ($\mu$mol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>0.09</td>
<td>5.92</td>
</tr>
<tr>
<td>Acetoadaldehyde</td>
<td>5.8</td>
<td>1.54</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>47</td>
<td>0.09</td>
</tr>
<tr>
<td>Isobutyaldehyde</td>
<td>6.2</td>
<td>0.75</td>
</tr>
<tr>
<td>n-Propanol</td>
<td>50</td>
<td>0.28</td>
</tr>
<tr>
<td>Aryl alcohol</td>
<td>100</td>
<td>0.05</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>10.6</td>
<td>3.67</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>30</td>
<td>0.36</td>
</tr>
<tr>
<td>n-Pentanol</td>
<td>6.5</td>
<td>4.24</td>
</tr>
<tr>
<td>Isopentanol</td>
<td>4.2</td>
<td>4.20</td>
</tr>
<tr>
<td>n-Hexanol</td>
<td>4.0</td>
<td>3.56</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>5.5</td>
<td>0.10</td>
</tr>
</tbody>
</table>

The enzyme reactions were carried out in 20 mM sodium carbonate–bicarbonate buffer, pH 8.9, for aldehyde substrates and in the same buffer but pH 10.8 for alcohol substrates.

The enzyme was inert toward the following aldehydes and alcohols: n-butyraldehyde, n-valeraldehyde, methanol, ethanol, isopropanol, sec-butanol, tert-butanol, and tert-pentanol.

Substrate specificity

The enzyme is NAD$^+$-dependent and does not require glutathione. The apparent $K_m$ and $V_{max}$ values for dehydrogenation of aldehydes and alcohols were estimated with a fixed, saturated concentration of NAD$^+$ and are summarized in Table II. For the aldehyde series, formaldehyde was the best substrate for the enzyme, the $K_m$ value being 0.09 mM and $V_{max}$ 5.92 $\mu$mol/min/mg. The $K_m$ value markedly increased and $V_{max}$ decreased with an increase in the length of the alkyl group. No activity was observed for tert-butanol and tert-pentanol, indicating that a length of three carbon atoms without a side-chain at the $\alpha$-carbon of the alkyl group is a minimum requirement for a substrate of the enzyme. The $K_m$ value for NAD$^+$ in dehydrogenation of aldehydes and alcohols was also examined.

Expected from the large $K_m$ values for alcohols (Table II), high concentrations were required for the maximum activity, especially with low molecular weight alcohols. No activity was observed for methanol or ethanol. Interestingly, $K_m$ values for alcohols tended to decrease parallel with the increase in the length of the alkyl group. This suggests that hydrophobicity of the alkyl group is related to the binding of an alcohol substrate to the enzyme. On the contrary, the $V_{max}$ value was variable: it rose with an increase in length of the alkyl group until n-pentanol and then decreased gradually. The enzyme was inert toward isopropanol, sec-butanol, tert-butanol and tert-pentanol, indicating that a length of three carbon atoms without a side-chain at the $\alpha$-carbon of the alkyl group is a minimum requirement for a substrate of the enzyme. The $K_m$ value for NAD$^+$ in dehydrogenation of aldehydes and alcohols was also examined.

*2 This value is different from that reported previously. We did not find that borate buffer used previously at pH 8~10 was markedly inhibitory for the dehydrogenase activity. On changing the buffer to a sodium carbonate–bicarbonate system, a value of 8.9 was obtained as the optimal pH for dehydrogenation of aldehydes.
Almost the same order of $K_m$ (0.16 ~ 0.39 mM) was obtained for all substrates (Table III).

### Inhibition of dehydrogenase activities for formaldehyde and n-butanol by other aldehydes and alcohols

Inhibitory effects of $n$-alcohols and aldehydes on the enzymatic dehydrogenation of formaldehyde and $n$-butanol were examined. As shown in Table IV, the activity for formaldehyde was competitively inhibited by the $n$-alkyl aldehyde series, whereas it was inhibited noncompetitively by a series of $n$-alkyl alcohols. On the other hand, the dehydrogenase activity for $n$-butanol was competitively inhibited by ethanol and $n$-propanol. Interestingly, however, the type of inhibition by aldehydes for $n$-butanol dehydrogenation was dependent upon the number of carbon atoms in the alkyl group; noncompetitive for acetoaldehyde but competitive for $n$-butyraldehyde and $n$-valeraldehyde. These results suggest that the binding sites for aldehydes and alcohols are somewhat different but are located fairly close to each other. Presumably, the hydrophobic pocket of the enzyme is occupied by both $n$-butanol and aldehydes of longer alkyl groups, though the latter compounds are unable to act as substrates for the enzyme. Steady-state kinetic analysis of the $Bi$ substrates–$Bi$ products reaction of this enzyme as well as the reaction mechanism of the dehydrogenation is now under investigation in detail and will be reported elsewhere.

### Acknowledgments

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### REFERENCES