An Induced Aliphatic Aldehyde Dehydrogenase from the Bioluminescent Bacterium, Beneckea harveyi

PURIFICATION AND PROPERTIES*

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A NAD$^+$-dependent aldehyde dehydrogenase, the activity of which induces at the same time as luciferase, has been purified from the bioluminescent bacterium Beneckea harveyi, and its chemical and physical properties have been investigated. The purification is accomplished in three steps resulting in an enzyme preparation that gives a single protein band on three different gel electrophoresis systems. The molecular weight of the purified enzyme was estimated to be 120,000 by gel filtration. Sodium dodecyl sulfate-gel electrophoresis gave a molecular weight of 59,000 indicating that aldehyde dehydrogenase has a dimeric structure with subunits of similar molecular weight. The purified enzyme has a high specificity for long chain aliphatic aldehydes; the Michaelis constants for aldehydes decrease with increasing chain length as also observed for bacterial aldehyde dehydrogenases involved in the metabolism of hydrocarbons. The aldehyde specificity of the aldehyde dehydrogenase is similar to that of luciferase indicating that the functional role of the enzyme may be linked with the bioluminescent system.

The metabolism of long chain aliphatic aldehydes in bioluminescent bacteria is of particular interest since these compounds are substrates for the bioluminescent reaction catalyzed by luciferase (1-5). Recently, we have reported the presence of an aldehyde dehydrogenase in crude extracts of the bioluminescent bacterium, Beneckea harveyi, with a high specificity for long chain aliphatic aldehydes (6). Although aldehyde dehydrogenases and their role in metabolism have been studied in a wide variety of organisms, most of these enzymes either have a broad specificity or do not function with the long chain aliphatic aldehydes (7). Only in a few microorganisms have aldehyde dehydrogenases with such specificity been observed (8-10). In these cases, the enzyme is induced directly by growth in the presence of the aldehyde, its hydrocarbon or alcohol analogue, or is found constitutively in an organism which grows on these compounds.

In contrast, the synthesis of aldehyde dehydrogenase in B. harveyi occurs without the addition of exogeneous long chain aliphatic compounds to the medium. The activity of aldehyde dehydrogenase in this bioluminescent bacterium may be under the same control as luciferase since low levels of activity are observed at early stages of bacterial growth followed by an increase in enzyme activity at the same time as the induction of luciferase (6). This result, as well as the similar specificity of aldehyde dehydrogenase and luciferase for long chain aliphatic aldehydes, suggests that the aldehyde dehydrogenase may be directly involved in the bioluminescent system of B. harveyi. In the present study, the purification of this enzyme and the characterization of some of its catalytic and structural properties are reported.

EXPERIMENTAL PROCEDURES

Materials—The commercial sources of reagents and chromatography media were the following: aldehydes, Aldrich; pyridine nucleotides and dithiothreitol, Sigma; guanidine hydrochloride, Mann ultracentrifuge; mercaptoethanol, cyagen bromide, diecylhydroxyfloro diimide, 6-amino-hexanoic acid, acrylamide, and bisacrylamide, Eastman; DEAE-cellulose 23, Bio-Rad; Sephrose 4B, Pharmacia; Ultrogel AcA-44, LKB; Aquasol II, Calbiochem. Proteins used as molecular weight standards were obtained from Sigma and Worthington. Luciferase was purified according to the method of Gunsalus-Miguel et al. (11). Phosphate buffers were prepared by mixing appropriate amounts of 1.0 M NaH$_2$PO$_4$ and 1.0 M K$_2$HPO$_4$.

Aldehyde Dehydrogenase Assay—Aldehyde dehydrogenase activity was measured by the increase in absorbance with time at 340 nm after addition of an aliquot of the enzyme to 1.0 ml of 0.05 M phosphate buffer containing 0.0022% dodecanal (10$^{-4}$ M) and 1.5 x 10$^{-6}$ M NAD$^+$. Aldehyde stock solutions (0.2%) were prepared in either 2-propanol or dimethylformamide. The units of activity in micromoles/min were calculated using a molar extinction coefficient of 6220 (0.1%, 1 cm) for NADH at 340 nm (12).

Protein Determinations—The protein concentration was generally measured by the method of Lowry (13) using bovine serum albumin as a standard. Alternatively, the protein was determined by the biuret method (14). Similar results were obtained (+10%) by both methods.

Dry Weight Measurements—Aldehyde dehydrogenase was dialyzed exhaustively against distilled deionized water, lyophilized, and then dried under vacuum over phosphorus pentoxide. The amount of protein in the sample (1.2 mg) was determined by the biuret method and compared to the dry weight (1.25 mg).

Amino Acid Analyses—Weighed samples of aldehyde dehydrogenase, prepared as above for dry weight measurements, were hydrolyzed in vacuo in 6 N HCl for 24, 48, and 72 h at 110°C. In some cases, an internal standard of norleucine was added initially to the HCl. After hydrolysis, the sample was dried on a flash evaporator, dissolved in citrate buffer, pH 2.2, and the amino acid composition
Purification and Properties of Aldehyde Dehydrogenase

Enzyme Purification – The steps for the purification of aldehyde dehydrogenase from the bioluminescent bacterium, Beneckea harveyi, are summarized in Table I. A detailed description of each step in the procedure is given in the miniprint supplement immediately following this paper.1

The first step of purification involves DEAE-cellulose chromatography of the enzyme from 30 g, wet weight, of cells. The enzyme was obtained by polyacrylamide gel electrophoresis in Tris/glycine, pH 8.9, and phosphate, pH 7.0, under nonde-}

**Table I**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume*</th>
<th>Total protein*</th>
<th>Total activity†</th>
<th>Specific activity</th>
<th>Yield</th>
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<tbody>
<tr>
<td>ml</td>
<td>mg</td>
<td>µmol/min</td>
<td>µmol/min/ mg</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Lysate supernatant</td>
<td>550</td>
<td>1890</td>
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<td>100</td>
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<tr>
<td>DEAE-cellulose</td>
<td>400</td>
<td>102</td>
<td>282</td>
<td>2.8</td>
<td>73</td>
</tr>
<tr>
<td>Chromatography</td>
<td>NH₄SO₄ fractionation</td>
<td>8.8</td>
<td>47</td>
<td>211</td>
<td>4.5</td>
</tr>
<tr>
<td>NAD⁺-Sepharose</td>
<td>60</td>
<td>9.8</td>
<td>144</td>
<td>14.7</td>
<td>37</td>
</tr>
<tr>
<td>Chromatography</td>
<td>Concentration by aquacide</td>
<td>1.8</td>
<td>7.9</td>
<td>158</td>
<td>17.3</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>11.4</td>
<td>4.5</td>
<td>79</td>
<td>17.7</td>
<td>21</td>
</tr>
</tbody>
</table>

* Volume of sample after the purification step.
† Protein concentrations were determined by the method of Lowry (13).
‡ Activity was determined as described under “Experimental Procedures” using 0.002% dodecanal in the assay.

aldehyde dehydrogenase activity in the final step of gel filtration. Due to the low capacity of the NAD⁺-Sepharose column, repeated attempts to adsorb the aldehyde dehydrogenase activity directly from the crude extract onto NAD⁺-Sepharose were unsuccessful.

The present procedure requires 3 days to obtain about 5 mg of aldehyde dehydrogenase from 30 g, wet weight, of cells. The final purification is less than 100-fold from the crude extract, suggesting that this enzyme comprises more than 1% of the solubilized protein, an amount comparable to luciferase (19, 20). The enzyme can be stored in phosphate buffer, pH 7.0, with 10% glycerol and 10⁻³ M dithiothreitol at 4°C for a period of 2 months without significant loss of activity. In the absence of glycerol, the enzyme lost 80 to 90% activity within 2 weeks.

Evidence for Homogeneity – Polyacrylamide gel electrophoresis of aldehyde dehydrogenase in sodium dodecyl sulfate (Fig. 1) shows a single polypeptide band. Since the molecular weight estimation by sodium dodecyl sulfate-gel electrophoresis and gel filtration are different (see below), this result suggests the aldehyde dehydrogenase is homogeneous since any contaminants must not only have the same molecular weight but also the same subunit structure.

Additional evidence for the homogeneity of the purified enzyme was obtained by polyacrylamide gel electrophoresis in Tris/glycine, pH 8.9, and phosphate, pH 7.0, under non-denaturing conditions. A single protein band was obtained in both cases (see miniprint supplement, Figs. 4S and 5S). In addition, it was shown that aldehyde dehydrogenase activity was directly associated with the single protein band in the phosphate gel (Fig. 5S).

Physical and Chemical Properties – The molecular weight of aldehyde dehydrogenase was estimated to be 120,000 by gel filtration on a column of Ultrogel AcA-44 calibrated with standards of known molecular weight assuming that the enzyme has the same shape and partial specific volume as the standard proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the enzyme along with standards of known polypeptide molecular weight gave an estimate of 59,000 for the molecular weight of the polypeptide chains of aldehyde dehydrogenase. These results indicate that aldehyde dehydrogenase is a dimer with subunits of identical molecular weight.

The absorption spectrum of aldehyde dehydrogenase had
Kₙ for aldehydes decreases about 1.5-fold for the addition of and NADP⁺ apparently has a much lower Kₙ than NAD⁺. Activities for AcPyAD and NADP⁺ are not due to differences in Kₙ since AcPyAD and NAD⁺ have comparable Kₙ values. AcPyAD and NAD⁺, and no activity with NMN. The lower activity with NAD⁺, with only 3 to 5% of this activity with NMN. These problems have been avoided in the present investigation by using higher NAD⁺ concentrations (1.5 mM) and by preparation of the stock aldehyde solutions in isopropyl alcohol or dimethylformamide.

Preparation of the stock aldehyde solutions at higher concentrations in 0.1% Triton X-100. The difficulty in using detergents for preparation of aldehyde dehydrogenase. The enzyme was applied to a 10% polyacrylamide gel in 0.1% sodium dodecyl sulfate, 0.01 M phosphate, pH 7.0, electrophoresed at 8 mA/gel at room temperature and stained for protein as described under "Experimental Procedures." The bottom of the gel indicates position of the tracking dye. an A₄₉₀/A₅₆ₕ ratio of 1.7 in phosphate, pH 7.0, with no absorption in the visible region of the spectrum. A specific absorption coefficient of 0.75 (0.1%, 1 cm) at 280 nm was calculated for aldehyde dehydrogenase using the biuret method to measure the protein concentration. The amount of protein determined by the biuret or Lowry methods (13) using bovine serum albumin as a standard was in close agreement with the dry weight of aldehyde dehydrogenase (98 and 106%, respectively), indicating that nonprotein components are not present in significant amounts.

Amino Acid Composition – The amino acid composition of aldehyde dehydrogenase is given in Table II. The total weight of amino acid residues agrees within 10% of the dry weight of the analyzed sample. An apparent partial specific volume of 0.732 ml/g was calculated from the amino acid composition (21). No similarity in amino acid composition was found between aldehyde dehydrogenase and luciferase (19), both of which catalyze reactions involving the oxidation of aliphatic aldehydes in this bioluminescent bacterium.

Enzyme Specificity – The specificity of aldehyde dehydrogenase for pyridine nucleotides is given in Table III. The purified enzyme catalyzes the reduction of NAD⁺ in the presence of aliphatic aldehydes with more than three carbons, whereas no activity is detected with propanal, acetaldehyde or a variety of aromatic aldehydes. The enzyme activity with aldehydes from butanal to tetradecanal is relatively constant with somewhat higher activities obtained with hexanal and heptanal. The specificity of the enzyme for long chain aliphatic aldehydes is reflected primarily in the decrease in Kₙ, as the chain length of the aldehydes is increased. The Kₙ for aldehydes decreases about 1.5-fold for the addition of each methylene group up to octanal and then more sharply (3- to 5-fold for each methylene group) at longer chain lengths.

The specificity for aliphatic aldehydes is somewhat different than previously reported for this enzyme in crude extracts. We have now reinvestigated the aldehyde dehydrogenase activity in crude extracts and found the lower activities reported earlier (6) were due to the low NAD⁺ concentrations (7 x 10⁻⁴ M) as well as incomplete solubilization of aldehyde stock solutions at higher concentrations in 0.1% Triton X-100. The difficulty in using detergents for preparation of aldehyde stock solutions has also been shown by Lebeault et al. (8). These problems have been avoided in the present investigation by using higher NAD⁺ concentrations (1.5 mM) and by preparation of the stock aldehyde solutions in isopropyl alcohol or dimethylformamide.

The specificity of aldehyde dehydrogenase for pyridine nucleotides is also given in Table III. The enzyme has maximum activity with NAD⁺, with only 3 to 5% of this activity with AcPyAD and NADP⁺, and no activity with NMN. The lower activities for AcPyAD and NADP⁺ are not due to differences in Kₙ since AcPyAD and NAD⁺ have comparable Kₙ values and NADP⁺ apparently has a much lower Kₙ than NAD⁺.

### Table II

<table>
<thead>
<tr>
<th>Amino Acid Composition of Aldehyde Dehydrogenase</th>
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<tbody>
<tr>
<td>Amino Acid</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Tryptophan</td>
</tr>
<tr>
<td>Cysteic acid</td>
</tr>
</tbody>
</table>

¹ The values shown are the average of duplicate analyses of 24, 48, and 72 h hydrolysates for three separate preparations of aldehyde dehydrogenase. The number of amino acid residues was calculated on the basis of a subunit molecular weight of 60,000.
² Expressed as a percentage of the total amino acid residues.
³ Determined after performic acid oxidation.
⁴ Determined after performic acid oxidation.
⁵ Determined by spectrophotometrically.
⁶ Determined after performic acid oxidation.

### Table III

<table>
<thead>
<tr>
<th>Substrate Specificity of Aldehyde Dehydrogenase</th>
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</thead>
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<tr>
<td>Aldehydes</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Acetaldehyde</td>
</tr>
<tr>
<td>Propanal</td>
</tr>
<tr>
<td>Butanal</td>
</tr>
<tr>
<td>Pentanal</td>
</tr>
<tr>
<td>Hexanal</td>
</tr>
<tr>
<td>Heptanal</td>
</tr>
<tr>
<td>Octanal</td>
</tr>
<tr>
<td>Nonanal</td>
</tr>
<tr>
<td>Decanal</td>
</tr>
<tr>
<td>Undecanal</td>
</tr>
<tr>
<td>Dodecanal</td>
</tr>
<tr>
<td>Tetradecanal</td>
</tr>
<tr>
<td>Benzaldehyde</td>
</tr>
<tr>
<td>Acetylbenzaldehyde</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
</tr>
<tr>
<td>p-Pentylbenzaldehyde</td>
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</table>

<table>
<thead>
<tr>
<th>Pyridine Nucleotides</th>
<th>10⁴ x Kₙ</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD⁺</td>
<td>3.6</td>
<td>100</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>&lt;0.01</td>
<td>100</td>
</tr>
<tr>
<td>AcPyAD⁺</td>
<td>3.1</td>
<td>3</td>
</tr>
<tr>
<td>NMN</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ Activity relative to dodecanal at saturating aldehyde and 1.5 mm NAD⁺.
² Activity relative to NAD⁺ at saturating pyridine nucleotide.
³ Activity relative to NAD⁺ at saturating pyridine nucleotide and 0.062% dodecanal (10⁻⁴ M).
⁴ 3-Acetylpyridine adenine dinucleotide.

### Discussion

A number of aldehyde dehydrogenases from microorganisms grown on long chain hydrocarbons show a similar specificity for long chain aliphatic aldehydes to the Beneckea...
harveyi enzyme. Two aldehyde dehydrogenases, inducible by growth on hydrocarbons, are found in Pseudomonas aerugi-
nosa, one of which is soluble and the other membrane bound
(10). Both enzymes have a similar specificity to the B. harveyi
enzyme for aliphatic aldehydes and the soluble enzyme shows
a decrease in $K_m$ for aldehydes with increasing chain length.
The $K_m$ values for aldehydes, however, are 10 to 100 times
higher than those reported in our study and decrease in a
relatively uniform fashion, only 1.2- to 1.3-fold for the addition
of each methylene group between butanal and tetradecanal.
A similar specificity and $K_m$ dependence for aldehydes has
been observed for constitutive membrane-bound aldehyde
dehydrogenase found in the marine bacterium, Alcaligenes
sp. (9). This latter strain was believed to have adapted to
growth on hydrocarbons released into the ocean by a nearby
oil refinery. In the yeast, Candida tropicalis, the specificity
of the aldehyde dehydrogenase for long chain aliphatic alde-
hydes is based on an increase in the maximum velocity with
aldehyde chain length rather than a change in $K_m$ (8). In
fact, the $K_m$ for aldehydes actually increases with increasing
chain length for this aldehyde dehydrogenase.

The organisms listed above can grow on hydrocarbons and
thus contain a defined system for hydrocarbon metabolism,
consisting of a hydroxylase and long chain alcohol and alde-
hyde dehydrogenases (9). In contrast, B. harveyi cannot grow
on hydrocarbons, and other enzyme constituents in this
system, such as a long chain alcohol dehydrogenase, have not
been detected in this organism. The aldehyde dehydrogenase
activity in R. harveyi is induced without the addition of
exogenous hydrocarbon derivatives by a yet unknown mech-
anism, increasing at the same stage of cell growth as the
luciferase activity (6). Double label experiments (22) have
confirmed that the increase in activity of luciferase is due to
protein synthesis (22) and have shown that other polypeptides
are also specifically synthesized during this period, including
a polypeptide that is present in a partially purified prepara-
tion of aldehyde dehydrogenase.

The enzymic basis for the in vivo synthesis of the aldehyde
required for the bioluminescent reaction has not yet been
demonstrated. Cline and Hastings (24) have shown that at
least two activities are involved in the production of the aldehyde or functionally equivalent factor (aldehyde factor) of
the bioluminescent reaction in B. harveyi. We have investi-
gated the possibility that aldehyde dehydrogenase could be
used to produce aldehyde by attempting to reverse the reaction
catalyzed by the enzyme. No decrease in the absorbance of
NADH could be obtained in the presence of ATP (+ Mg$^{2+}$),
alddehyde dehydrogenase and long chain aliphatic acids or

* A. Bognar and E. Meighen, unpublished experiments.

A definitive role can not be assigned to aldehyde dehydro-
genase in this organism at the present stage of study. The
limited aldehyde specificity of this enzyme, having no activity
with aromatic and short chain aldehydes, would make it of
little use as a general detoxifier to remove aldehydes from the
cell (7). It appears that the function of this enzyme may be
to specifically remove the aldehyde (or aldehyde factor)
produced as substrate for the bioluminescent reaction. It will
be of interest therefore to determine what specific role alde-
hyde dehydrogenase plays in the bioluminescent system.

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Additional references can be found on p. 450.
Supplement to
An Induced Alkaline Aldehyde Dehydrogenase from the Staphylococci
Bacterium, Review of Lamps, Purification and Properties.

by A. Lamp and O. Helgum

Method of Preparation of NAD

Separation of NAD was accomplished by a method of Lamps and Helgum [1] described in detail by Lamp et al. [2]. The first step in the purification procedure was the preparation of a crude enzyme extract. This was done by the addition of 0.1 M phosphate buffer, pH 7.0, to the bacterial cells. The mixture was then centrifuged at 10,000 rpm for 30 minutes and the supernatant fluid was collected. The crude enzyme extract was then subjected to a column of DEAE-cellulose. The column was washed with 0.1 M phosphate buffer, pH 7.0, and then eluted with a gradient of 0.1 to 1.0 M NaCl. The enzyme activity was found in the fraction eluted at 0.5 M NaCl.

Procedures for the purification of NAD

The purified enzyme was subjected to gel filtration on a Sephadex G-200 column. The elution profile was monitored by measuring the optical density at 280 nm. The enzyme activity was found in the fraction eluted at 0.5 M NaCl.

Physical Properties

The enzyme has a molecular weight of approximately 45,000, as determined by gel filtration on a Sephadex G-200 column. The enzyme is stable at pH 7.0 and is active over a pH range of 6.5 to 8.0. The enzyme is inhibited by dithiothreitol and is activated by NAD.

References

An induced aliphatic aldehyde dehydrogenase from the bioluminescent bacterium, *Beneckea harveyi*. Purification and properties.

A L Bognar and E A Meighen


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