Journal of Industrial Microbiology & Biotechnology (2000) 25, 328–332

www.nature.com/jim

The carboxylic acid reduction pathway in *Nocardia*. Purification and characterization of the aldehyde reductase

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Whole cultures of *Nocardia* sp. NRRL 5646 reduce carboxylic acids, first to aldehydes, then to alcohols and subsequently to the corresponding acetyl esters. This work describes an NADPH-dependent reductase responsible for catalyzing the reduction of aldehyde intermediates, which was purified 3240-fold by a combination of Mono-Q, hydroxyapatite, and ADP-agarose chromatographies. By sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the purified enzyme ran as a single band of 47 kDa. A native molecular mass estimated at 101 kDa indicated that the enzyme was a homodimer in the native, active state. Edman degradation indicated a unique N-terminal sequence as NH_2-X-X-Ala-Ala-Ala-Tyr-Ala-Val-Pro-Ala-Pro-Asp-Gly-Cys-Phe-Glu-Lys-Val-Thr-Ile-Glu-Arg-Arg-Glu-Leu-Gly. The enzyme catalyzed reductions of many aryl- and alkyl-aldehyde substrates. Reactions were most favorable in the direction of aldehyde reduction to alcohols. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 328–332.

Introduction

We have described the capabilities of cultures of *Nocardia* species NRRL 5646 to efficiently reduce a wide range of carboxylic acids (Figure 1). The reduction reaction occurs sequentially, and it involves at least three separate enzymes. These enzymes are a carboxylic acid reductase that converts carboxylic acids into aldehydes [22–24,31], an aldehyde reductase that converts aldehydes into alcohols, and an acyl transferase that catalyzes the formation of acetyl esters of the alcohol products [7]. The efficient conversion of carboxylic acids to alcohols or their corresponding acetyl esters is an attractive whole-cell reaction sequence for the biocatalytic synthesis of fragrances and flavors, and for pharmaceutical intermediates. With whole cultures of *Nocardia*, the carboxylic acid reduction process is enantioselective as shown in the reduction of isomers of ibuprofen [7]. The requirement for NADPH, the reducing cofactor, is readily satisfied by *in situ* biosynthesis by growing cultures during whole-cell biocatalysis.

Properties of the first enzyme involved in carboxylic acid reduction in *Nocardia* were completely established by enzyme isolation and purification studies, and by analysis of the mechanism of the reduction process [22,23]. Purified *Nocardia* carboxylic acid reductase catalyzes two major reactions: an ATP-dependent conversion of carboxylic acids to acyl adenylates and the subsequent NADPH-dependent reduction of acyl adenylate intermediates to aldehydes [22,23]. In this paper, we describe the purification, characterization and properties of the *Nocardia* aldehyde reductase, the enzyme that catalyzes the second step in the reaction sequence shown in Figure 1.

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Received 8 May 2000; accepted

Materials and methods

Materials

2′,5′-ADP-agarose was purchased from Sigma Chemical (St. Louis, MO). Mono-Q anion exchanger was purchased from Pharmacia (Piscataway, NJ). Hydroxyapatite and molecular standards for gel electrophoresis were obtained from Bio-Rad (Hercules, CA). All other reagents were purchased from Sigma Chemical or Aldrich Chemical (Milwaukee, WI).

Preparation of cell free extracts

Nocardia species NRRL 5646 is maintained in the University of Iowa, College of Pharmacy culture collection. The organism was grown and maintained on slants of Sabouraud-dextrose agar or sporulation agar (ATCC No. 5 medium). Shaken flash cultures were grown by a standard two-stage incubation protocol [4] in 200 ml of sterile soybean flour–glucose medium held in stainless-steel-capped, 1-l DeLong culture flasks. The medium contained (wt/vol) 2% glucose, 0.5% yeast extract, 0.5% soybean flour, 0.5% NaCl and 0.5% K_2HPO_4 in distilled water and was adjusted to pH 7.2 with 6 N HCl before being autoclaved at 121°C for 20 min. Cultures were incubated by shaking them at 250 rpm at 28°C on New Brunswick Scientific, G25 Gyrotory shakers. A 10% inoculum derived from a 72-h-old, first-stage culture was used to initiate the second-stage culture, which was incubated as before. *Nocardia* cells were harvested 48 h after second-stage culture inoculation, by centrifugation at 8000×*g* for 20 min, and washed twice with 0.9% NaCl. Cell pellets were stored at −38°C until needed. Typical wet cell yields by this cultivation process were 25 g/l.

For the preparation of cell free extracts, 25 g (wet weight) of cell pellet was suspended in 150 ml of cold 50 mM, Tris–HCl buffer (pH 7.5) containing 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 10% (v/v) glycerol. The cell suspension was disrupted with a Sonifier Cell Disrupter 350 (Branson Sonic Power, Danbury, CT) operating at full power at 250 W with a 20% intermittent duty cycle for 10

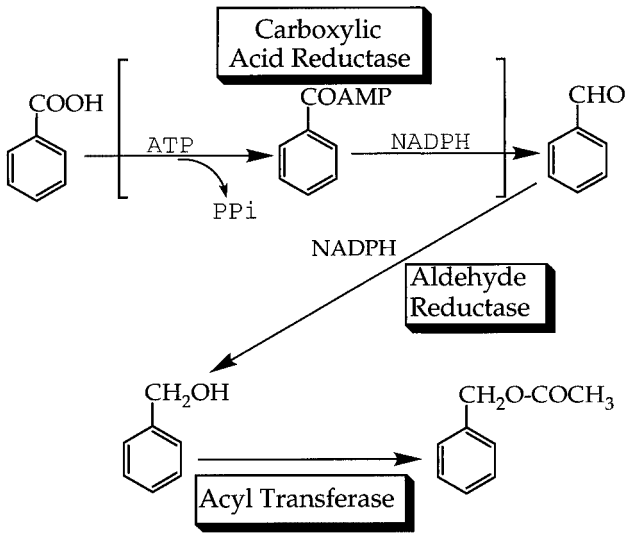

Figure 1 Pathway for reduction of benzoic acid by *Nocardia* sp. NRRL 5646.

min. Cell debris was removed by centrifugation in a Beckman L8-55 ultracentrifuge at 100,000×*g* for 40 min at 4°C. The 100,00×*g* supernatant was directly used for subsequent enzyme purification steps, which were all conducted at 4°C.

Enzyme assay

The standard aldehyde reductase assay solution contained 0.15 mM NADPH, 1 mM benzaldehyde, and 0.01 to 0.3 units of enzyme in 50 mM pH 7.5 Tris–HCl buffer in a final volume of 0.7 ml. For spectrophotometry, the reference cuvette contained all components except for benzaldehyde.

Reactions were initiated by adding enzyme, and they were monitored for 1–2 min by recording the absorption decrease at 340 nm at 25°C with a Shimadzu 160 spectrometer as the results of NADPH oxidation (ε=6400 M^{-1} cm^{-1}). One unit of the aldehyde reductase is defined as the amount of enzyme that catalyzes the reduction of 1 μmol benzaldehyde to benzyl alcohol per minute under standard assay conditions.

Protein assay

The concentration of protein was measured by the Bradford protein microassay [6], with bovine serum albumin as the standard.

Enzyme purification

Crude 100,000×*g* supernatant (150 ml, approximately 160 mg protein) was applied to a Mono-Q column (2×20 cm) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF and 10% (v/v) glycerol. The column was then washed with 60 ml of starting buffer before the enzyme was eluted with a 0–1 M NaCl linear gradient (400 ml) in starting buffer while 5-ml fractions were collected. The active fractions (fractions 22 to 28) were combined and 40 mg of $MgCl_2 \cdot 6H_2O$ were added before hydroxyapatite chromatography.

The preparation was loaded onto a hydroxyapatite column (2×8 cm) equilibrated with 5 mM phosphate buffer, pH 6.8. The column was then washed with 40 ml of starting buffer. Enzyme was eluted with a linear gradient from 5 to 100 mM phosphate buffer, pH 6.8 (300 ml) while fractions of 3.8 ml were collected. The active fractions (fractions 52 to 64) were combined and concentrated to 5 ml with an Amicon concentrator (YM10 membrane). The solution was diluted to 50 ml with 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM DTT, 10 mM $MgCl_2$, and 10% (v/v) glycerol and concentrated to 5 ml by the same concentrator.

The enzyme was loaded onto an ADP-agarose column (0.7×10 cm) pre-equilibrated with the same buffer used for enzyme dilution in the preceding step. The column was washed with 30 ml of starting buffer and then the enzyme was eluted with 100 ml of the Tris–HCl buffer with a linear gradient of $NADP^+$ (0–2.5 mM) while 1.5-ml fraction were collected. The active fractions (fractions 13 to 35) were combined and concentrated with an Amicon concentrator (YM 10 membrane) for subsequent analysis.

SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Bio-Rad Mini-Protein II dual-slab cell with a discontinuous buffer system [21] and a 10% separation gel. Gels were stained with Coomassie Blue. Protein standards used for estimation of subunit molecular masses were myosin (208 kDa), β-galactosidase B (107 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (79.5 kDa), ovalbumin (49.5 kDa), and carbonic anhydrase (29 kDa).

Native molecular weight

Analytical gel filtration chromatography was carried out with an Alltech Macrosphere 150 column (7 μm, 0.46 by 25 cm). The mobile phase of 0.3 M phosphate buffer, pH 7.0 was used to equilibrate the column and to elute protein samples at a flow rate of 0.2 ml/min. Eluted protein peaks (retention volumes, R_v) were monitored at 280 nm. The standards were Blue dextran (2000 kDa, R_v=1.8 ml), apoferritin (443 kDa, R_v=1.9 ml), β amylase (200 kDa, R_v=2.1 ml), aldehyde reductase (150 kDa, R_v=3.1 ml), phosphorylase b (97.4 kDa, R_v=3.3 ml), and carbonic anhydrase (29 kDa, R_v=4.1 ml).

N-terminal amino acid sequence

N-terminal amino acid sequence analysis was determined by Edman degradation in an automated sequencer at the University of Iowa, Protein Structure Facility.

Absorption spectrum

The UV–visible absorption spectrum of the aldehyde reductase (30 μg in 0.1 ml of 50 mM Tris–HCl buffer, pH 7.5) was recorded with a Sim-Aminco model DW2000 UV–visible spectrometer with 0.1 ml cuvettes. The spectrum was scanned over the range of 200 to 600 nm.

Substrate specificity

Purified enzyme substrate specificity was evaluated in incubations using 1 mM concentrations of various aldehyde substrates in the same buffer used for enzyme assays. Relative rates of aldehyde reductions were expressed as a percentage of the initial rate observed with benzaldehyde reduction (Table 2).

Kinetic studies

Evaluation of the kinetic mechanism for benzaldehyde reduction with NADPH was carried out in 50 mM Tris–HCl (pH 7.5). Initial

rate patterns were analyzed with software based on the Fortran programs of Cleland [9]. The kinetic mechanism was established by comparing the fitting variances of experimental data to several different models, and confirmed by graphic analyses. Product inhibition studies with either benzyl alcohol or $NADP^+$ were performed to confirm the kinetic model [13]. Kinetic parameters were estimated with the same software once the mathematical model was chosen.

Results

Enzyme purification

The results of a typical purification of *Nocardia* aldehyde reductase are summarized in Table 1. For protein stabilization, DTT, PMSF, and EDTA were routinely included in buffers, and 10% glycerol was necessary to prevent enzyme inactivation in all subsequent steps.

In the Mono-Q ion-exchange step, the enzyme was eluted between 180 and 400 mM NaCl. $MgCl_2$ was added to pooled active fractions to permit enzyme binding to hydroxyapatite in the next step. This enabled direct loading of the active Mono-Q fractions without desalting or buffer exchange. The enzyme eluted from the hydroxyapatite column between 60 and 80 mM phosphate buffer. After rapid buffer exchange, the enzyme preparation was then subjected to ADP agarose affinity chromatography where it was eluted between 0.5 mM and 1.0 mM $NADP^+$ to give an enzyme preparation (SDS-PAGE, Figure 2, lane 2) suitable for further study. Overall, the aldehyde reductase was purified 3240-fold from the crude extract with a specific activity of 295 units per milligram of protein.

Aldehyde reductase properties

While the enzyme retained more than 90% of its activity at 30°C for 4 h in crude extracts, it was much less stable during later stages of purification. Enzyme activity was determined in a variety of buffers (phosphate, Tris–HCl, and Gly–NaOH) over a pH range of 6–10. The enzyme showed highest activity at pH 8.2 in Tris–HCl. Enzyme assays were conducted at pH 7.5 (90% of the activity at pH 8.2) to favor enzyme stability. Highest enzyme activity was measured at 50°C in pH 7.5, 50 mM Tris–HCl. However, enzyme activity in this buffer was completely lost in less than 30 min at 45°C. The enzyme required NADPH for aldehyde reductions, and was inactive with NADH.

By Alltech Macrosphere 150 gel filtration, the active enzyme was eluted at 3.11 ml ($n=3$), to give an estimated native molecular mass of 101 kDa. By SDS-PAGE, the denatured molecular mass was estimated to be 47 kDa (Figure 2). UV–visible spectroscopy

Table 1 Purification of an aldehyde reductase from *Nocardia* sp. NRRL 5646

Steps	Total protein (mg)	Total activity (Units)	Specific activity (u/mg)	Yield (%)	Purification (fold)
Crude extract	160	14.6	0.09	100	1
Mono-Q	55.3	15.0	0.27	103	3
Hydroxyapatite	9.3	8.4	0.91	58	10
ADP-Agarose	0.026	7.8	295	54	3240

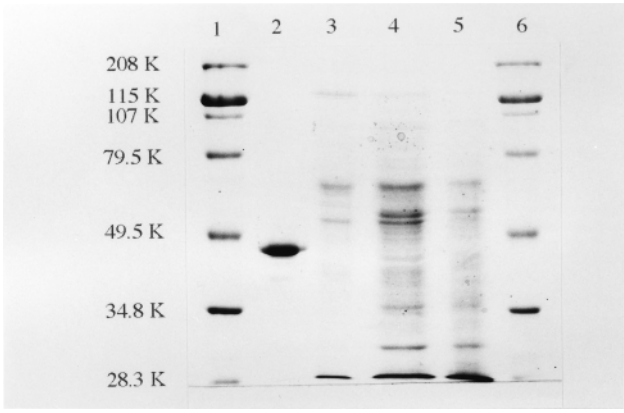

Figure 2 SDS-PAGE of samples from the purification of *Nocardia* sp. NRRL 5646 aldehyde reductase. Lanes 1 and 6, protein markers; lane 2 purified aldehyde reductase; lane 3, hydroxyapatite fraction; lane 4, Mono-Q fraction; lane 5, crude extract.

of the native enzyme showed an absorption maximum at 282 nm. Edman degradation gave an N-terminal sequence of the enzyme as NH_2-X-X-Ala-Ala-Ala-Tyr-Ala-Val-Pro-Ala-Pro-Asp-Gly-Cys-Phe-Glu-Lys-Val-Thr-Ile-Glu-Arg-Arg-Glu-Leu-Gly.

Relative activities of the reductase versus a range of aldehydes as measured by the consumption of NADPH in the presence of enzyme plus substrate are summarized in Table 2. In general, substituted benzaldehydes were reduced at rates similar to that for benzaldehyde. The influence of regiosubstitution on the aromatic ring on aldehyde reduction rates was most apparent with anisaldehyde where the 3- and 2-isomers were reduced at 120% and 18% versus benzaldehyde, respectively. In general, for electron-donating or neutral aromatic ring substituents, 3-substituted benzaldehydes were the best substrates. Nitrobenzaldehyde

Table 2 Substrate specificity of *Nocardia* aldehyde reductase

Substrate (1 mM)	Relative activity[a]
Benzaldehyde	100
2-Fluorobenzaldehyde	110
3-Fluorobenzaldehyde	105
4-Fluorobenzaldehyde	90
2-Chlorobenzaldehyde	96
3-Chlorobenzaldehyde	109
4-Chlorobenzaldehyde	91
2-Bromobenzaldehyde	68
3-Bromobenzaldehyde	112
4-Bromobenzaldehyde	111
2-Anisaldehyde	18
3-Anisaldehyde	120
4-Anisaldehyde	84
3-Hydroxybenzaldehyde	16
4-Nitrobenzaldehyde	4
D,L-Glyceraldehyde	1.5
D-Glucuronic acid	0
Pyridine-3-aldehyde	18
Phenylacetaldehyde	21
2-Phenylpropanaldehyde	39
Propanaldehyde	33
Acetophenone	0.5

[a]Benzaldehyde was reduced at a rate of 36.6 μM min^{-1} with a ΔOD min^{-1} of 0.228.

and glyceraldehyde were poor substrates. Phenylacetaldehyde, phenylpropanaldehyde and propanaldehyde were similarly reduced. Glucuronic acid was not a substrate, and acetophenone reduction was at 0.5% the rate of benzaldehyde reduction. The enzyme catalyzed the reverse reaction, but the rate of benzyl alcohol oxidation to benzaldehyde at 30 mM was 4.9 ± 0.8 μmol min^{-1} (mg protein)$^{-1}$ ($n=3$), less than 1% of that for benzaldehyde reduction.

Reciprocal plots of initial rate data obtained when both NADPH and benzaldehyde substrate concentrations were varied were subjected to regression analysis. The data gave best fit with the least variance for a sequential enzyme mechanism [9,33]. Michaelis constants for NADPH and benzaldehyde were calculated to be 20.0 ± 2.3 and 12.1 ± 1.5 μM, respectively [9]. The order of substrate binding and product release during aldehyde reduction was determined by product inhibition. $NADP^+$ was a competitive inhibitor to NADPH, and a noncompetitive inhibitor to benzaldehyde. Benzyl alcohol showed noncompetitive inhibition patterns to both benzaldehyde and NADPH.

Discussion

Reductions of carboxylic acids to alcohols occur in many types of microorganisms including *Actinomyces* [16], *Clostridium thermoaceticum* [39], *Aspergillus niger* [2,29], *Corynespora melonis* [2], *Coriolus* species [2], *Neurospora crassa* [3,14], *Glomerella cingulata* [34,35], *Gloeosporium laeticolor* [35] and *Nocardia* species [7,18,19,26]. However, properties of the reducing enzymes are not generally well known. We earlier characterized the carboxylic acid reductase enzyme system of *Nocardia* sp. NRRL 5646 [22,23], and were interested in establishing the properties of the aldehyde reductase responsible for the second step in carboxylic acid reductions to alcohols (Figure 1). Understanding the properties of each of the enzymes involved in serial reactions in whole-cell conversions is essential to establishing useful whole-cell biocatalytic processes.

The enzyme purification described here was highly reproducible, recovering 54% of the total activity found in crude, $100{,}000 \times g$ supernatants. The most important component of the purification process was the 2′,5′-ADP agarose affinity step, which also works very well with other NADPH requiring enzymes [8]. Pure enzyme was obtained by a 320-fold purity increase with ADP-agarose.

The homodimeric aldehyde reductase is a soluble, NADPH-dependent enzyme. BLASTP and TBLASTN program searches of the updated SwissProt, Genpept, GenBank and EMBL databases gave no matching amino acid sequences [1]. The UV spectrum shows normal protein absorbances indicating the lack of prosthetic groups in the enzyme, such as flavins or heme. The enzyme reduces a wide range of substrates including aliphatic, aromatic and heterocylic aldehydes. Interestingly, the range of aldehyde substrates reflects the range of carboxylic acid substrates for the first enzyme in the reduction pathway (Figure 1). Modest reactivity with glyceraldehyde indicates a substrate similarity with mammalian aldose reductase (EC 1.1.1.21) [5,12,37]. Lack of activity with D-glucuronic acid indicates a lack of similarity to mammalian aldehyde reductase (EC 1.1.1.2) [5,10–12,36].

Crude cell free extracts of many microorganisms contain NADPH-dependent aldehyde reductases [17,40]. Although a few microbial enzymes are relatively specific for the reductions of aldehydes to alcohols [15,20,40], most NAD[P]-dependent enzymes [30] favor the reverse reaction of alcohol dehydrogenation much like that observed with liver alcohol dehydrogenase (EC 1.1.1.1) [28].

The *Nocardia* enzyme shows relatively high activities for substituted benzaldehydes, suggesting substrate specificities similar to NADPH-dependent cinnamyl alcohol dehydrogenases (CADs) (EC 1.1.1.195), which are involved in substituted cinnamaldehyde reductions during lignin biosynthesis in plants [25]. Interestingly, CADs are homodimeric proteins with subunit weights of about 40 kDa [27,32]. The N-terminal sequence of the *Nocardia* enzyme was unique, showing no homology to CADs or any other known sequences.

The *Nocardia* aldehyde reductase is different than liver alcohol dehydrogenase (EC 1.1.1.1) because it greatly favors aldehyde reduction versus alcohol oxidation by a different kinetic mechanism [28]. Glyceraldehyde is a very poor substrate thus indicating that the *Nocardia* enzyme does not belong to the aldose reductase family (EC 1.1.1.21) [5]. The *Nocardia* aldehyde reductase failed to reduce glucuronic acid, indicating another difference versus mammalian aldehyde reductases (EC 1.1.1.2). The K_m for benzaldehyde is about 10-fold less than that for typical mammalian aldehyde reductases [10–12,36–38].

Initial rate and product inhibition patterns for the *Nocardia* aldehyde reductase are consistent for an ordered Bi Bi mechanism [33] in which NADPH binds to the enzyme first, followed by benzaldehyde. After reduction of benzaldehyde, benzyl alcohol is released from the enzyme as the first product, and $NADP^+$ as the last reaction product. Product inhibition patterns observed with the *Nocardia* enzyme ruled out mechanisms for either Theorell-Chance or rapid equilibrium random Bi Bi processes, both of which give the same initial rate equation as for an ordered Bi Bi mechanism [13,33].

These studies have defined the properties of a new NADPH-dependent aldehyde reductase that catalyzes the second step in whole-cell biocatalytic reductions of carboxylic acids to alcohols and esters. The substrate range for the enzyme is broad, and it favors reductions of aldehydes to alcohols versus the reverse action. Understanding the properties of enzymes involved in carboxylic acid reduction (Figure 1) provides a functional basis for designing useful whole-cell biocatalyst processes with *Nocardia* sp. NRRL 5646.

Acknowledgements

Tao Li acknowledges support through a Center for Biocatalysis and Bioprocessing Fellowship. We are grateful for financial support from the USDA through the Byproducts for Biotechnology Consortium. Grateful thanks to Professor B.V. Plapp, Department of Biochemistry, College of Medicine, University of Iowa for providing the enzyme kinetic model software.

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