A Low Molecular Weight Fungal Alcohol Dehydrogenase Requiring NADPH

JULIAN BETTER and STEN GATENBECK

Department of Biochemistry and Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden

During the studies of the biosynthesis of barnol (4,6-dimethyl-5-ethylpyrogallol) in *Penicillium baarnense* it was found that aryl-alcohol dehydrogenases played an important role in the biosynthetic reaction sequence.[1]

In an investigation of aryl-alcohol dehydrogenases present in *P. baarnense* several enzymes catalyzing the reduction of aryl-aldehydes have been detected. In the following the isolation and characterization of one of them will be described.

Experimental. Organism and growth conditions. Penicillium baarnense v. Beyma CBS 315.59 was obtained from *Centraalbureau voor Schimmelcultures,* Netherlands, and it was subcultured on malt agar slants. Spore suspensions were transferred to 1 000 ml conical flasks containing 350 ml of Raulin-Thom medium consisting of glucose (50 g), ammonium tartrate (2.6 g), tartaric acid (2.6 g), yeast extract (1.0 g), $(NH_4)_2HPO_4$ (0.4 g), $(NH_4)SO_4$ (0.2 g), K_2CO_3 (0.4 g), $MgCO_3$ (0.3 g), $FeSO_4$ $7H_2O$ (0.05 g), $ZnSO_4$ $7H_2O$ (0.05 g), and distilled water (total volume 1 000 ml). The cultures were grown at 28 °C on a rotary shaker until barnol appeared in the culture medium (3–4 d). The mycelium from the organism was harvested by filtration at reduced pressure and it could be stored at −20 °C for a week without losing enzyme activity.

Enzyme assay. During the purification the alcohol dehydrogenase was routinely assayed by following the decrease of NADPH absorbance at addition of benzaldehyde in a Gilford 2600 spectrophotometer. The assay was performed at 25 °C in 50 mM potassium phosphate buffer, pH 7.2, supplemented with 10 mM 2-mercaptoethanol, 0.1 M EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM NADPH, and 30 mM benzaldehyde. Total volume was 1 ml including 50 µl enzyme extract.

Enzyme purification. The mycelium (100 g wet weight) was subjected to disintegration at 4 °C in a glass bead mill with 100 ml of the buffer described in the assay. Centrifugation at 25 000 g for 20 min gave a supernatant to which was added two portions of each 150 mg protamine sulfate. Recentrifugation after each addition at 48 000 g for 20 min yielded a final clear red coloured solution. The volume of this crude enzyme extract was reduced by ultrafiltration (Diaflo XM-50, Amicon) to about 25 ml. The concentrated enzyme solution was fractionated by gel filtration on Ultrogel AcA34 (IBF, France). The fractions containing the aryl-alcohol dehydrogenase investigated were pooled and subjected to dye-ligand chromatography (Matrex Gel Red A, Amicon). The column was freed from unbound proteins by washing with 1 M phosphate buffer, pH 7.3, followed by 50 mM phosphate buffer of the same pH. The enzyme was eluted with 0.15 mM NADP in 50 mM phosphate buffer. The purification procedure is summarized in Table 1.

Results and discussion. The homogeneity of the purified enzyme was checked by analytical isoelectric focusing using a pH range of 3.0–9.0 as described by Wrigley.[2] Only one band appeared (isoelectric point 4.6) when the gel was developed for enzyme activity with the method described by Hillmer and Gottschalk[3] using benzyl alcohol as substrate or for protein content with Coomassie-Brilliant Blue G250.

The molecular weight of the dehydrogenase was found to be close to 30 000 as estimated by gel chromatography (Sephadex G-150, Superfine, Pharmacia) using the following proteins as markers: Chymotrypsin (M=25 000), ovalbumin

Table 1. Purification of the enzyme.

Step	Total protein (mg)	Total activity (U)	Recovery (%)	Specific activity (U/mg)
Crude extract	1 108	110	–	0.1
Protamine sulfate precipitation	988	98	89	0.1
Ultragel AcA34	18	14	13	0.8
Matrex Gel Red A	0.4	5.2	5	13.0

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Table 2. Kinetic parameters of the enzyme.[a]

Substrate	V_{max} (mmol · s^{-1} · mg enzyme^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat/K_m} (s^{-1} · mM^{-1})
Benzaldehyde	0.30	35	9 000	257
3-Hydroxybenzaldehyde	0.23	12	6 900	575
Butyraldehyde	0.12	10	3 600	360
Caproaldehyde	0.33	10	9 900	990
Dihydroxyacetone	0.82	1	24 600	24 600
Crotonaldehyde	0.04	3.5	1 200	343
Cinnamic aldehyde	0.16	2.3	768	334

[a] Measurements were made at 25 °C in 50 mM potassium phosphate buffer of 7.3 with NADPH at an initial concentration of 0.2 mM. Substrate concentrations ranges 0.13–35 mM were used.

(M=45 000), bovine serum albumin (M=68 000), lactoperoxidase (M=85 000), tyrosinase from mushroom (M=125 000). The molecular weight was confirmed by dodecylsulfate acrylamide electrophoresis according to the method of Weber and Osborn.[4] The electrophoresis also revealed the absence of subunits. The low molecular weight and the monomeric structure are noteworthy characteristics of a phosphopyridine nucleotide requiring dehydrogenase.

The enzyme uses as coenzyme NADPH that cannot be substituted with NADH. When benzaldehyde is the substrate the pH optimum of the reaction is 7.5. The substrate specificity and some kinetic parameters of the enzyme are shown in Table 2. Even though the aromatic aldehydes benzaldehyde and 3-hydroxybenzaldehyde are good substrates for the enzyme aliphatic aldehydes serve as substrates as well. Dihydroxyacetone seems to be a particularly suitable substrate and it cannot be excluded that it is the natural substrate of the enzyme. Apparently, like many other pyridine nucleotide requiring dehydrogenases the isolated enzyme has a broad specificity. The reversibility of the reduction reactions was shown by oxidation of the alcohols corresponding to the aldehydes listed in Table 2.

The enzyme activity is inhibited irreversibly by the presence of *o*-iodosyl-benzoic acid or iodoacetic acid. The inhibition by *p*-hydroxymercuribenzoate is reversible. The participation of the described enzyme in the biosynthesis of barnol is not likely as the reduction of such aldehydes as 2-hydroxy-3-methoxy-6-methylbenzaldehyde, 2-hydroxy-3-methoxy-5,6-dimethylbenzaldehyde, 2,4-dihydroxy-6-methylbenzaldehyde, and 2,4-dihydroxy-6-ethyl-5-methylbenzaldehyde are not catalyzed by the enzyme.

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