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Properties of Diacetyl (Acetoin) Reductase from *Bacillus stearothermophilus*

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Abstract—The cells of *Bacillus stearothermophilus* contain an NADH-dependent diacetyl (acetoin) reductase. The enzyme was easily purified to homogeneity, partially characterised, and found to be composed of two subunits with the same molecular weight. In the presence of NADH, it catalyses the stereospecific reduction of diacetyl first to (3*S*)-acetoin and then to (2*S*,3*S*)-butanediol; in the presence of NAD^+, it catalyses the oxidation of (2*S*,3*S*)- and *meso*-butanediol, respectively, to (3*S*)-acetoin and to (3*R*)-acetoin, but is unable to oxidise these compounds to diacetyl. The enzyme is also able to catalyse redox reactions involving some *endo*-bicyclic octen- and heptenols and the related ketones, and its use is suggested also for the recycling of NAD^+ and NADH in enzymatic redox reactions useful in organic syntheses. Copyright © 1996 Elsevier Science Ltd

Introduction

Acetoin (acetylmethyl carbinol) is a major metabolic product in some bacteria and yeast species and an important metabolite of tumor cells.[1] *Enterobacter aerogenes*, a pathogen Gram-negative bacterium, contains an enzyme (EC 1.1.1.5) able, in the presence of NADH, to reduce diacetyl (2,3-butanedione) first to L(+)-acetoin and then to L(+)-2,3-butanediol; the same enzyme catalyses, in the presence of NAD^+, the oxidation of this last compound to L(+)-acetoin;[2–8] this enzyme is composed of two subunits with a molecular weight of 28 kDa.[8] Other diacetyl reductases require NADPH.[9–15] Acetoin is also a substrate of multienzyme systems with dihydrolipoamide and thiamine diphosphate coenzymes.[16,17]

We report now the purification to homogeneity and a partial characterisation of an NADH-dependent diacetyl (acetoin) reductase from non-pathogenic *B. stearothermophilus* . We have also found that this enzyme is the secondary alcohol dehydrogenase recently discovered[18] to be able to catalyse efficiently and stereospecifically redox reactions involving some *endo*-bicyclic octen- and heptenols and related ketones, reagents useful for the organic synthesis of many compounds with important biological roles. This enzyme, for its characteristics, can be used in organic synthesis for the preparation of enantiomerically pure compounds.

Key words: Diacetyl reductase, acetoin reductase, butanediol dehydrogenase, *Bacillus stearothermophilus*.

Results

Purification of the enzyme

Table 1 shows the results of a typical purification procedure. The enzymatic activity was measured using NADH and racemic acetoin as reagents. The purification procedure required less than 5 h, had a 46% yield, and starting from 15 g of wet cells gave 6.3 mg of enzyme after a 62-fold purification. The purified enzyme was homogeneous, as detected by the presence of a single band in the SDS–PAGE, and was not contaminated by ethanol or lactate dehydrogenase activities.

Table 1. Purification of *B. stearothermophilus* diacetyl (acetoin) reductase

Fractions	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	75	977	860	1.14	100	1
DEAE sepharose column	79	458	136	3.37	47	2.9
Blue agarose column	2.5	450	6.3	71.4	46	62.6

Molecular weight of the enzyme and of its subunits

The molecular weight of the enzyme, determined by size exclusion chromatography (Fig. 1A) was 49 kDa. With SDS–PAGE we detected a single protein band with a molecular weight of 26 kDa (Fig. 1B). Thus, the enzyme is composed of two subunits with an equal molecular weight.

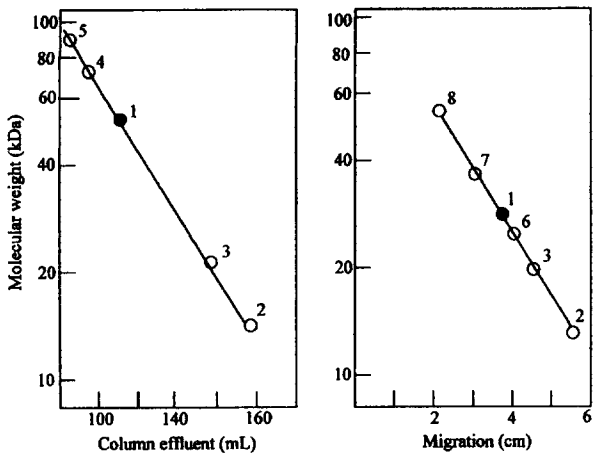

Figure 1. Determination of the molecular weight of the enzyme and of its subunits. Panel A: size exclusion chromatography on Ultragel ACA 44. Panel B: SDS–PAGE. The position of the diacetyl (acetoin) reductase is indicated by No. 1. The reference proteins were: (2) cytochrome C; (3) soybean trypsin inhibitor; (4) serum albumin; (5) yeast alcohol dehydrogenase; (6) trypsin; (7) rabbit muscle lactate dehydrogenase; (8) 6-phosphogluconate dehydrogenase.

Stability of the enzyme

The purified enzyme was unstable to dilution; kept diluted at 0 °C for ca. 60 min lost 62% of activity (Fig. 2). This inactivation was almost completely reversed by the addition of NAD^+. To avoid inactivation, the enzyme was kept at 0 °C in the presence of 20% glycerol, 0.1 mM EDTA, 5 mM β-mercaptoethanol and 0.6 mM NAD^+ in TEA buffer, pH 7.5. In these conditions, the enzyme (at a protein concentration of 3 mg/mL) had a half-life of 1 month; instead kept for 3.5 h at 40 °C, only 10% of its original activity was lost. The enzymatic reactions were carried out at 25 °C; due to the presence of NAD^+, the enzyme in the reaction mixture did not significantly decrease its activity at this temperature for at least 10 h. In the case of the reduction of acetoin to butanediol, the reaction rate reached a maximum at 50 °C. The enzyme was inactivated by ammonium sulfate precipitation.

Substrates, reactions, and stereospecificity

The reactions catalysed by the enzyme are reported in Figure 3. Using diacetyl as substrate, NADH is oxidised (Fig. 3, reaction 1) with a specific activity of 26.3 mmol/min/mg of protein with the transient formation of (3*S*)-acetoin. Starting from racemic acetoin, there was oxidation of NADH with a specific activity of 71.4 and the formation of both (2*S*,3*S*)-butanediol and *meso*-butanediol (Fig. 3, reactions 2 and 3). These findings are supported by the stoichiometry of the reactions: in the presence of enzyme, each molecule of diacetyl is able to oxidise two molecules of NADH, indicating that it is first reduced to acetoin and then to butanediol. The addition of enzyme to a solution containing NADH (300 nmol/mL), (3*S*)-acetoin and (3*R*)-acetoin (each 100 nmol/mL; a racemic mixture), caused the formation of 200 nmol of NAD^+, indicating that both enantiomers are reduced.

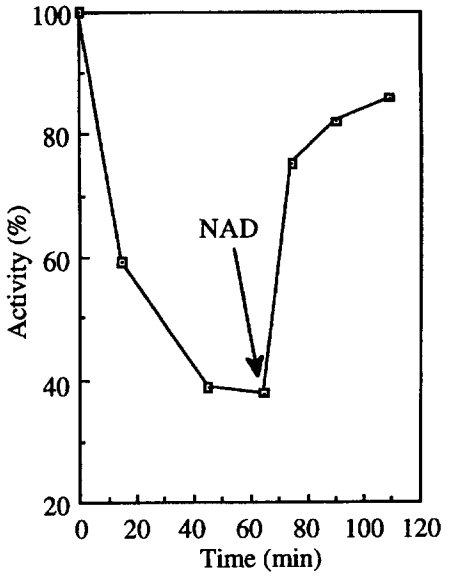

Figure 2. Reactivation of the enzyme by NAD^+. The purified enzyme was diluted in 50 mM TEA buffer, 0.1 mM EDTA, and 2 mM β-mercaptoethanol (pH 7.5) to a final protein concentration of 0.03 mg/mL. After 60 min, the solution was made 0.6 mM in NAD^+. Samples were removed at determined time intervals and analysed for enzymatic activity.

Using as substrate (2*S*,3*S*)-butanediol or *meso*-butanediol, NAD^+ was reduced with specific activities of 44.9 and 14.8, respectively. There was the formation of (3*S*)- or (3*R*)-acetoins, respectively (Fig. 3, reactions 4 and 5). The enzyme, in the presence of NAD^+ and at pH 9.2, was unable to catalyse the oxidation of (2*R*,3*R*)-butanediol or racemic acetoin (5 mM). The redox reactions required the presence of a diphosphopyridine dinucleotide, the triphosphopyridine dinucleotides being ineffective.

Kinetic properties

The rate of the enzymatic reactions was affected by the pH of the reaction mixture. The pH optimum was 6.5 for the reduction of diacetyl and 9.2 for the oxidation of (2*S*,3*S*)-butanediol (Fig. 4). The values (obtained from Lineweaver–Burk plots) of the K_m of the substrates and of V_{max} of these reactions are reported

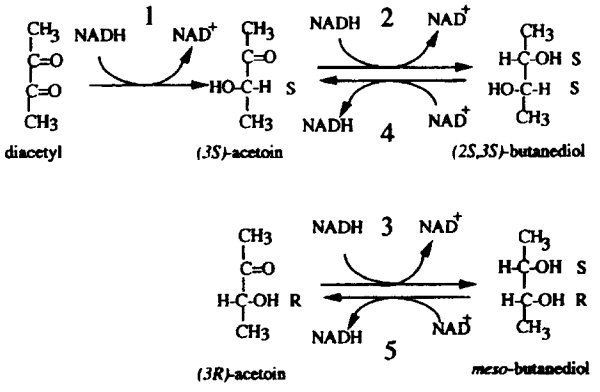

Figure 3. Reactions catalysed by the diacetyl (acetoin) reductase from *B. stearothermophilus*.

Table 2. Kinetic parameters of the reactions

Substrate	Coenzyme	pH	Approx K_m (mM)	V_{max} (U/mg)
Diacetyl	NADH	6.5	19	30.1
Racemic acetoin	NADH	6.5	2.2	81.4
(2*S*,3*S*)-Butanediol	NAD^+	9.2	1.2	51.9
6-*endo*-Bicyclo[3.2.0]-hep-5-en-6-ol	NAD^+	9.2	0.6	36
Bicyclo[3.2.0]-hep-5-en-6-one	NADH	6.6	4.4	0.7

in Table 2. In these experiments the concentrations of NADH and NAD^+ were 0.16 and 0.45 mM, respectively; and the concentrations of the substrates ranged from 0.15 to 1.5 mM in the case of (2*R*,3*R*)-butanediol; from 1.2 to 12.5 mM in the case of acetoin; and from 2 to 20 mM in the case of diacetyl. The K_m for NAD^+, using 10 mM (2*S*,3*S*)-butanediol as substrate, was 14 μM.

The enzyme-catalysed reaction between NAD^+ and (2*S*,3*S*)-butanediol, at pH 9.2, did not go to completion, but reached an equilibrium. Starting from different concentrations of (2*S*,3*S*)-butanediol and measuring the concentrations of (2*S*,3*S*)-butanediol, (3*S*)-acetoin, NAD, and NADH in the equilibrium conditions, we calculated that the equilibrium constant, at pH 9.2, was 1.32×10^{-10} M.

Activities on bicyclo compounds

We recently reported[18] that *B. stearothermophilus* contain an enzyme which catalyses stereospecific redox reactions, also in semipreparative scale, of some bicyclo alcohols and ketones. We have now found that this enzyme is the diacetyl (acetoin) reductase described in this report. In Table 2 we report the values of kinetic parameters, obtained with this purified enzyme, using as substrate 6-*endo*-bicyclo[3.2.0]-hep-5-en-6-ol and bicyclo[3.2.0]-hep-5-en-6-one.

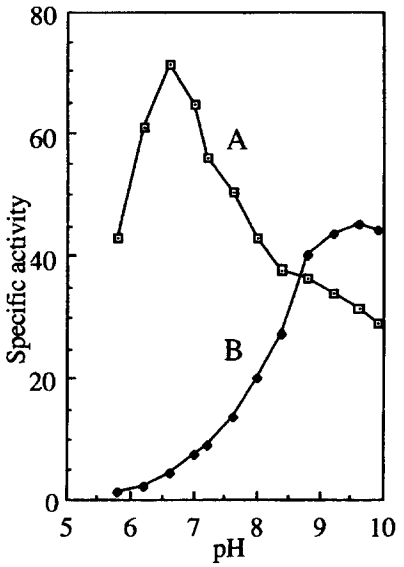

Figure 4. Effect of pH on the rate of the reactions. The buffers used were 50 mM phosphate (pH 5.8–8.0) or 50 mM DEA (pH 8.0–10), both containing 0.1 mM EDTA. The reduction reactions (line A) were carried out in presence of 0.18 mM NADH and 5 mM racemic acetoin. The oxidation reactions (line B) were carried out in the presence of 0.4 mM NAD^+ and 5 mM (2*S*,3*S*)-butanediol.

Discussion

The diacetyl (acetoin) reductase described here can be purified easily to homogeneity from cells of *B. stearothermophilus* in less than 5 h and with a good yield. The protein is composed of two subunits with the same molecular weight and some of its kinetic parameters have been determined. In the presence of NAD or NADH the enzyme catalyses stereospecifically the reactions reported in Figure 3. At the right pH values, the rates of the reactions of oxidation and of reduction are of the same order of magnitude, thus the enzyme catalyses both types of reactions equally well.

Using the GLC we have determined the absolute configuration of the chiral centres of the substrates and of the products of the reactions catalysed by the enzyme and seen that only the hydroxy groups bound to an (*S*)-carbon atom are involved in the oxidation reactions and that reductions give only (*S*)-carbon atoms. Thus, the enzyme follows the Prelog rule[19] as yeast, horse liver and *Thermoanaerobium brokii* alcohol dehydrogenases and carbonyl reductases from *Candida parapsilosis* and *Rhodococcus erythropolis*.[20]

The oxidation reaction of (2*R*,3*R*)-butanediol has a pH optimum of 9.2 (with half-maximal rates at pH 8.2 and at a value higher than 10.5), while the reduction of acetoin has a pH optimum of 6.2 (with half-maximal rates at pH 5.6 and 8.5). If these values were confirmed from the graphs of pK_m/pH, the amino acid residue with a p*K* of 8.2–8.5 could be a cysteine residue, (which could act, unprotonated, in the oxidation reaction and, protonated, in the reduction reaction) while the group with a p*K* of 5.6 could be a histidine residue.

We have now seen that this purified enzyme is the same enzyme recently found[18] to catalyse stereospecifically, in the presence of NAD^+ or NADH, redox reactions involving a series of bicyclic heptenols and -ones, important starting materials for the chemical synthesis of biologically active compounds (such as prostacyclin, prostaglandin, leukotrienes and sarkomycin, and pheromones). It appears that in the oxidation reaction there are little differences in both the K_m and V_{max} values between the 4-C atoms compounds and the bicyclo substrates; in the reduction reaction, instead, the V_{max} is significantly lower for the bicyclic ketone. It is surprising that this enzyme works almost equally well on substrates having such different structures. It has been isolated[21] from *T. brokii* an NADPH-dependent alcohol dehydrogenase which catalyses the reduction of these bicyclic ketones, but not the oxida-

tion of the related alcohols. This enzyme is used also for the recycling of $NADP^+$.

There is an ever increasing number of enzymes employed for the industrial synthesis of chiral compounds. If these reactions are redox and use pyridine dinucleotide coenzymes, these coenzymes have to be recycled and the best way is to use an oxidoreductase. The enzyme described here, catalysing easily both the reduction of NAD^+ and oxidation of NADH, and having as substrates inexpensive compounds either hydrophobic or hydrophilic, has the best requisites for this task. Furthermore, this easily obtained enzyme, can also be used to prepare enantiomerically pure (3*S*)- and (3*R*)-acetoins or (2*S*,3*S*) butanediol.

Materials and Methods

Diacetyl, (2*S*,3*S*)-butanediol, (2*R*,3*R*)-butanediol, *meso*-butanediol, and a racemic mixture of acetoins were Aldrich products. NAD^+, $NADP^+$, NADH, NADPH, egg white lysozyme, Cibachrom-blue 3GA agarose, and other fine chemicals were Sigma products. Lamb liver 6-phosphogluconate dehydrogenase was prepared as described.[22]

Enzyme assays

Enzyme assays were carried out at 22 °C by monitoring the absorbancy change at 340 nm of the diphosphopyridine dinucleotide coenzyme (NAD^+ or NADH) involved in the redox reaction. The reductions of diacetyl and acetoin were carried out in 50 mM phosphate buffer, pH 6.5, containing 0.1 mM EDTA, 0.16 mM NADH, and 5 mM substrate. The oxidation of 2,3-butanediol was carried out in 50 mM diethanolamine (DEA) buffer, pH 9.2, containing 0.1 mM EDTA, 0.4 mM NAD^+, and 5 mM substrate. The reactions were started by addition of few μL of enzyme to 1 mL of the reaction mixture and followed for 1 min taking readings at 0.1 min intervals. One unit (1 U) of enzyme activity is defined as the amount of enzyme that transforms 1 μmol of substrate/min under standard assay conditions. The specific activity is the ratio between enzymatic units and mg of protein.

Cell preparation

B. stearothermophilus (American Type Culture Collection, ATCC 2027) was grown in 250 mL of a medium composed of sucrose (10 g), peptone (5 g), yeast extract (2.5 g), $NaH_2PO_4 \cdot 6H_2O$ (1.7 g) and K_2SO_4 (0.65 g). Growth was carried out at 39 °C for 48 h with reciprocal shaking. Wet cells (15 g), obtained from four cultures, were harvested by centrifugation (8000 rpm, 15 min at 4 °C) and washed with 200 mL of ice cold 0.15 M NaCl.

Enzyme purification

The purification of the enzyme was followed measuring the reduction of acetoin. Protein concentrations were determined by the Bradford procedure[23] in the crude extract and by the absorbancy at 280 nm for purified enzyme, assuming that a solution containing 1 mg/mL of protein has an absorbancy of 1.00 at 280 nm.

Step 1. The cells of *B. stearothermophilus* (15 g) were suspended in 75 mL of 50 mM triethanolamine (TEA) buffer, pH 7.5 (containing 0.1 mM EDTA and 1 mM β-mercaptoethanol) and treated with 40 mg of lysozyme for 60 min at 25 °C. The suspension was centrifuged (15,000 rpm, 15 min at 4 °C) and the supernatant (75 mL) was used for the enzyme purification.

Step 2. The crude extract was chromatographed on a DEAE sepharose CL 6B column (11 × 2.5 cm) equilibrated with the buffer used for the extraction of the enzyme. The flow rate was 1 mL/min and 10 mL fractions were collected. The enzyme was not bound by the resin and was eluted with the equilibration buffer (*DEAE sepharose column*).

Step 3. The fractions containing enzyme activity were pooled and chromatographed on a column (6 × 3 cm) of Cibachrom-blue 3GA agarose equilibrated and washed with the above mentioned buffer at a rate of 1 mL/min, until no proteins were present in the effluent. The enzyme was eluted with 0.8 M NaCl (in the same buffer and at the same rate). The fractions containing enzymatic activity were pooled and concentrated to 2.5 mL using Centriflo CF25/CF50A membrane cones Amicon (*Blue agarose column*).

Molecular weight determinations

The molecular weight of the enzyme was determined by size exclusion chromatography at 4 °C on a column (2.8 × 40 cm) of Ultragel ACA 44, equilibrated with 50 mM phosphate buffer pH 7.5, 0.1 mM EDTA, 1 mM mercaptoethanol. The flow rate was 0.4 mL/min; fractions of 1 mL were collected and analysed for enzymatic activity or absorbancy at 280 nm. SDS–PAGE was made according to Laemmli.[24]

Gas–liquid chromatography

GLC analyses were performed with a Carlo Erba HR GLC 6000 Vega series, equipped with a chiral column Megadex 5 (25 m × 0.25 mm) containing *n*-pentyl, dimethyl, β-cyclodextrin in OV 1701 from Mega snc. The detection was carried out with a flame ionization detector and the separation was performed with a gradient of 2 °C/min from 80 to 200 °C with a helium flow of 1.5 mL/min. The reagents and products contained in the enzymatic reaction mixtures were lyophilised, and the compounds, extracted with ethylacetate, were analysed by GLC. The retention time of the substrates and of the products of the enzymatic reactions were as follows: diacetyl 2 min, (3*S*)-acetoin 3.8 min, (3*R*)-acetoins 4.1 min, (2*S*,3*S*)-butanediol 9.1 min and (2*R*,3*R*)-butanediol 9.5 min and *meso*-butanediol 10.1 min.

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