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Purification and characterization of the 1-3-propanediol dehydrogenase of Clostridium butyricum E5

Hassiba Malaoui, Régis Marczak*

Laboratoire de Biochimie des Bactéries Gram+, Domaine scientifique Victor Grignard, Université Henri Poincaré, Faculté des Sciences, BP 239, 54506 Vandoeuvre lès Nancy Cédex, France

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Abstract

1-3 PPD dehydrogenase (EC 1.1.1.202) was purified to homogeneity from Clostridium butyricum E5 grown anaerobically on glycerol in continuous culture. The native enzyme was estimated by gel filtration to have a molecular weight of 384 200 ± 31 100 Da; it is predicted to exist as an octamer or a decamer of identical molecular weight subunits. When tested as a dehydrogenase, the enzyme was most active with 1-3 propane diol. In the physiological direction, 3-hydroxypropionaldehyde was the preferred substrate. The apparent K_m values of the enzyme for 3-hydroxypropionaldehyde and NADH were 0.17 mM and 0.06 mM, respectively. The enzyme requires only Mn^{2+} for full activity. The enzyme was found to have properties similar to those reported for *Klebsellia pneumoniae*, *Citrobacter freundii*, and *Clostridium pasteurianum*. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Glycerol fermentation; Propane diol dehydrogenase purification; 3-hydroxypropionaldehyde; Clostridium butyricum

1. Introduction

Glycerol is available in large quantities as one of the products of saponification. It serves as a chemical feed stock for various purposes and is also available as a substrate for biotechnological processes. One product that can be readily manufactured from glycerol is 1.3-propanediol (1.3-PPD). Glycerol fermentation into 1.3-PPD was studied with facultative anaerobic microorganisms of the genera *Klebsiella* and *Citrobacter* [1] and with strictly anaerobic bacteria such as *Clostridium* [2].

Clostridium butyricum, a strictly anaerobic spore-forming bacterium is known as a classical acid producer and usually ferments carbohydrates to butyrate, acetate, carbon dioxide, and molecular hydrogen. The production of acetate or butyrate constitutes an important branch point of glycerol fermentation. *C. butyricum* is also able to catabolize glycerol using additional branch point. Indeed glycerol can be either oxidized by an NAD-linked glycerol dehydrogenase (dha D) to dihydroxyacetone (DHA) and subsequently phosphoryled to yield DHAP by dihydroxyacetone kinase (dha K) and funnelled to the central metabolism, or it can be dehydrated to 3-hydroxypropionaldehyde (3-HPA) by coenzyme B12-dependent glycerol dehydratase (dha B). The latter compound is then reduced to 1.3-PPD by the NADH-linked 1.3-PPD dehydrogenase (dha T). The key enzymes of glycerol fermentation are glycerol dehydrogenase and dihydroxyacetone kinase for oxidative branch and glycerol dehydratase and propanediol dehydrogenase for the reductive branch. The enzymes glycerol dehydrogenase, diol dehydratase, and 1.3-PPD dehydrogenase constitute the branch point that partitions the carbon flux between the competing pathways, i.e. formation of either 1.3-PPD or pyruvate [3]. The four key enzymes of this pathway are encoded by the dha regulon, the expression of which is induced when DHA or glycerol is present [4,5].

1.3-propanediol oxidoreductase (1.3-propanediol dehydrogenase, EC 1.1.1.202) was originally detected in extracts of glycerol-grown cells as an enzyme that catalyzes the oxidation of NADH at the expense of 3-HPA [6]. In contrast to the 1.3-PPD-forming enteric bacteria, only little is known about the enzymes responsible for glycerol breakdown by clostridia. The activities of glycerol dehydrogenase, glycerol dehydratase, and 1.3-PPD dehydrogenase have been determined in crude extracts of *C. butyricum* [7] and the latter activity in *C. pasteurianum* [8]. It is most likely that

* Corresponding author. Tel.: +33/3-83-91-25-51; fax: +33/3-83-91-25-50.

E-mail address: marczak@lcb.u-nancy.fr (R. Marczak).

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2.5. Molecular mass determinations

Molecular weight of the native enzyme was determined by two methods:

1. Gel filtration on a FPLC column of superose 12 HR 10/30 equilibrated with 50 mM KPB (pH 7.4) containing 100 mM KCl. Column was calibrated with known molecular weight standards (Da) (Sigma, St. Louis, MO, USA): blue dextran (2 000 000), apoferritin (443 000), alcohol dehydrogenase (150 000), human serum albumin (67 000), ovalbumin (45 000), cytochrome C (12 400).
2. Electrophoresis under nondenaturing conditions was carried out on 5% slab gels in Tris-glycine buffer (pH 8.3). Activity staining of 1.3-PPD dehydrogenase was performed as described by Boenigk [19] using crude extract of *C. pasteurianum* [15] and alcohol dehydrogenase as enzyme markers. For calculation of the native molecular mass, a high molecular-mass calibration kit of standard proteins was used (Da) (Sigma): thyroglobulin (669 000), apoferritin (443 000), β amylase (200 000), alcohol dehydrogenase (150 000). Proteins bands were located by staining with blue Coomassie.

The subunit size was estimated by submitting a part of the active protein fraction eluted from gel filtration to polyacrylamide gel electrophoresis under denaturing conditions. Electrophoresis was carried at room temperature. The samples were diluted 2× in SDS gel-loading buffer containing 100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue and 20% glycerol, boiled for 5 min, loaded into the well of a 12% denaturing gel, and subjected to electrophoresis with molecular weight standards (Bio–Rad) for approximately 45 min at a constant voltage of 200 volts. The following proteins were served as subunit molecular weight standards: conalbumin (76 000), albumin (66 200), actin (43 000), GAPDH (36 000), carbonic anhydrase (31 000), trypsin inhibitor (21 500), myoglobin (17 500). Proteins bands were located by staining with blue Coomassie.

2.6. Determination of the N-terminal amino acid sequence

The analysis was performed by the protein sequencing device of the Henri Poincaré University (Nancy, France).

After SDS-polyacrylamide electrophoresis of the purified enzyme and blotting onto a PVDF membrane (Prosorb), amino acid sequence analysis was performed on a 476 A microsequencer (Perkin–Elmer, Applied Biosystems Division, Foster City, CA, USA) with on-line identification of the phenylthiohydantoïn derivates.

The N-terminal amino acid sequence was determined by the use of the 610 A Data Analysis System (ABI).

2.7. Determination of the optimum pH

Assays to determine the optimum pH were performed with 0.2M KPB adjusted to the appropriate values with 3M KOH or 3M HCl. DL-Gld and 3-HPA were reduced in the presence of 0.37 mM NADH (10 mM DL-Gld or 3.5 mM 3-HPA was used). 1.3-PPD and glycerol were oxidized in the presence of 2 mM NAD^+ (100 mM 1.3-PPD or glycerol were used). The optimum pH values were calculated by nonlinear regression to the Bell-Shaped Double pKa equation by use of the Curve Fit feature of the program Grafit (Erithacus Software).

2.8. Determination of kinetic parameters

The apparent K_m values obtained with substrates and coenzymes were determined at 37°C with potassium carbonate buffer (pH 9.7 for the oxidative reactions and pH 9.1 for the reductive reactions). They were determined from the results of experiments in which a fixed concentration of the substrate or coenzyme and an appropriate range of concentrations of the other reactant were used.

The K_m value was expressed in millimolar and calculated by nonlinear regression to the Michaelis–Menten equation by use of the Curve Fit feature of the program Grafit.

2.9. Determination of substrate specificity

The activity of 1.3-PPD dehydrogenase in oxidation reactions was determined at 37°C spectrophotometrically at 340 nm by the initial rate of substrate-dependent NADH increase. The assay mixture contained 100 mM KCO_3 buffer (pH 9.7), 30 mM $(NH_4)_2SO_4$, 2 mM NAD^+, and 100 mM substrate in a 1-ml final volume. Activities were expressed relative to those obtained with 1.3-PPD.

The enzyme activity in reduction reactions was determined under the same assay conditions described in oxidation reactions, except that the assay mixture contained 100 mM KCO_3 buffer (pH 9.1), 30 mM $(NH_4)_2SO_4$, 0.37 mM NADH, and 10 mM substrate in a 1-ml final volume. Activities are expressed relative to those obtained with 3-HPA.

2.10. Determination of the effect of mono and divalent cations

The chloride salts of ammonium, sodium, potassium, magnesium, or lithium (10 mM) and iron, manganese or calcium (1 mM) were included with 100 mM 1.3-PPD, 2 mM NAD^+, and 100 mM carbonate buffer (pH 9.7) to determine the effects of these cations on enzyme activity.

3. Results

3.1. Enzyme purification

1.3-PPD dehydrogenase was purified from *C. butyricum* E5 by the procedure detailed in Section 2. The oxidoreductase believed to be responsible for the reduction of 3-HPA to 1.3-PPD was passed over a Q Sepharose ion exchange

Table 1
Purification steps of 1,3-propanediol dehydrogenase from *C. butyricum* E5[a]

Steps	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	115.2	99	0.86	1	100
Anion exchange chromatography	46.1	83	1.8	2.1	83.8
Gel filtration	0.41	3.8	9.3	10.8	38.4[b]

[a] The purification procedure is described in Section 2.
[b] The recovery was estimated by taking into account the sample volume injected (0.2 ml) versus the total volume obtained after ultrafiltration (2 ml).

nificant activity was detected with 1.2-propanediol, glycerol, 2.3-butanediol, ethylene-glycol, 2-propanol, glycerol-3-P. The reduction reaction was less specific. The enzyme was most active with 3-HPA but considerably less active with acetaldehyde, DHA, DL-Gld, and propionaldehyde (in that order of decreasing relative activity). No reduction of N-butyraldehyde was detected.

The apparent K_m values determined for various reactions catalyzed by this enzyme are summarized in Table 3. The enzyme exhibited Michaelis–Menten kinetics, the K_m values for NADH were approximately equivalent in the presence of either substrate (DL-Gld and 3-HPA). The K_m values for 3-HPA was 7-fold less than for DL-Gld and 20-fold less than for 1.3-PPD. This result showed that the affinity of the enzyme for its physiological substrate (3-HPA) is considerably higher than for DL-Gld and 1.3-PPD.

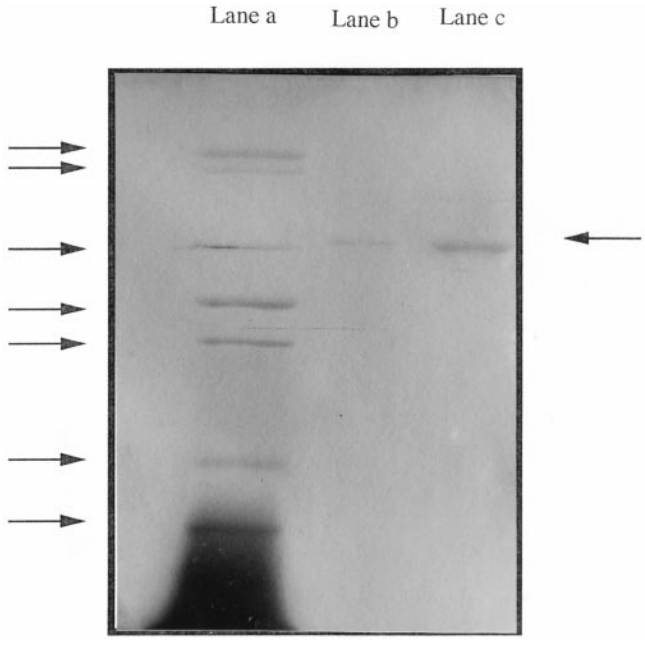

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified 1.3-PPD dehydrogenase. The purified protein was subjected to electrophoresis on a 12% polyacrylamide slab gel in the presence of 10% SDS. The proteins bands were stained with blue Coomassie. Lanes: a, molecular mass markers top to the bottom (conalbumin, 76 000; bovine serum albumin, 66 200; actin, 43 000; GAPDH, 36 000; carbonic anhydrase, 31 000; trypsin inhibitor, 21 500; myoglobin, 17 500); b and c, 2.05 and 4.10 μg of the purified 1.3-PPD dehydrogenase, respectively.

3.6. Effect of enzyme by mono and divalent cations

With respect to cations effect, the purified enzyme from *C. butyricum* exhibited the highest levels of activity (measured as the oxidation of 1.3-PPD) in the presence of 1 mM Mn^{2+}. Replacement of Mn^{2+} by other mono or divalent cations such as 10 mM Mg^{2+}, Na^{+}, Li^{+}, K^{+}, NH_4^{+}, or 1 mM Ca^{2+}, Fe^{2+} involved a 60 to 90% reduction of the relative activity. Among the cations tested, only Mn^{2+} stimulated the activity of 1.3-PPD dehydrogenase from *C. butyricum*.

4. Discussion

The 1.3-PPD dehydrogenase purified from *C. butyricum* was found by gel filtration to have a molecular mass of 384 200 Da. Activity staining of 1.3-PPD dehydrogenase on nondenaturing polyacrylamide gel electrophoresis indicated a molecular mass of 440 000 Da similar to the *C. pasteurianum* enzyme. Based on the denaturing polyacrylamide gel electrophoresis, the enzyme could contain eight identical subunits with molecular weight of 48 750 Da. Based on the 78% homology with *C. pasteurianum* enzyme and dhaT deduced gene product, the enzyme could also be a decamer of a polypeptide of 41 776 Da [15]. 1.3-PPD dehydrogenases have been purified from *L. brevis, L. buchneri* [11], *L. reuteri* [12], *K. pneumoniae* [10], *C. freundii* [13], and *C. pasteurianum* [15]. Molecular masses reported for the native enzyme are 180 000 Da for the *L. reuteri* enzyme, 440 000 Da for the *C. pasteurianum* enzyme, and around 350 000 Da for the other three enzymes. The molecular mass of the subunits varied between 41 000 Da and 46 000

M S Y R M F D Y L V P N V N	*K. pneumoniae*
M S Y R M F D Y L V P A N V N	*C. freundii*
M . . R M Y D F L A P N V N F M	*C. pasteurianum*
M . . R M Y D Y L V P S V N F M	*C. butyricum*

Fig. 4. N-terminal amino acid sequences alignement (12 to 14 amino acids) of the 1.3-PPD dehydrogenases purified from some strains of *Enterobacteria* and *Clostridia.*

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