Biochimica et Biophysica Acta, 445 (1976) 286–293

BBA 67896

L-RHAMNOSE DEHYDROGENASE OF *PULLULARIA PULLULANS*

LIU UN RIGO, MOMOYO NAKANO, L.A. VEIGA and DAVID SIDNEY FEINGOLD

Institute of Biochemistry, Federal University of Paraná, 80.000/Curitiba, Paraná (Brazil) and Department of Microbiology, School of Medicine, Univesrity of Pittsburgh, Pittsburgh, Pa. 15261 (U.S.A.)

(Received March 16th, 1976)

Summary

Growth of *Pullularia pullulans* on L-rhamnose induces formation of L-rhamnofuranose dehydrogenase, which reversibly converts L-rhamnofuranose to L-rhamnono-γ-lactone with the concomitant reduction of NAD, but not of NADP. The dehydrogenase was purified 100-fold by $MnCl_2$ treatment, $(NH_4)_2SO_4$ fractionation, Sephadex G-50 filtration, and fractionation on DEAE-cellulose. The pH optimum is 9.0, K_m for L-rhamnose is 0.2 mM and for NAD 0.02 mM. K_{eq} at pH 6.8 for the reaction L-rhamnose + $NAD^+ \rightleftharpoons$ L-rhamnono-γ-lactone + H^+ is 12 μM. The following could not replace L-rhamnose as substrate for the enzyme: D- or L-arabinose, D- or L-fucose, D-galactose, D-glucose, D- or L-mannose or D- or L-xylose. Extracts of L-rhamnose-grown *P. pullulans* also contain a lactonase which converts L-rhamnono-γ-lactone to L-rhamnonate.

Introduction

The 6-deoxyaldohexose L-rhamnose is utilized by *Escherichia coli* by initial isomerization to L-rhamnulose [1], and conversion of the latter to the 1-phosphate [2], which then is cleaved by L-rhamnulose 1-phosphate aldolase to yield dihydroxyacetone phosphate and L-lactaldehyde [3]. In 1961 Eagon [4] suggested the existence of a second pathway for 6-deoxyaldohexose metabolism in a number of bacterial species. This suggestion was substantiated by Dahms and Anderson [5] who showed that a soil pseudomonad utilized D-fucose by initial dehydrogenation to D-fuconate. The D-fuconate was subsequently dehydrated to 2-keto-3-deoxy-D-fuconate [6], which finally was cleaved to yield pyruvate and D-lactaldehyde [7].

Although the metabolism of 6-deoxyhexoses in bacteria is well known, little information is presently available concerning the utilization of these compounds in fungi. In this paper we show that the first step in the catabolism of L-rhamnose by the yeastlike fungus *Pullularia pullulans* is the NAD-linked dehydrogenation of L-rhamnofuranose to L-rhamnono-γ-lactone, which is hydro-

lyzed to L-rhamnonate by a specific lactonase. Preliminary evidence suggests that the L-rhamnonate is thereafter converted to 2-keto-3-deoxy-L-rhamnonate and that the latter is cleaved to pyruvate and L-lactaldehyde [8].

Materials and Methods

Materials

L-Rhamnonic acid was prepared by the method of Moore and Link [9]. L-Rhamnose (4 g) in 8 ml of distilled water was diluted with 50 ml of methanol and mixed with a solution of 11.4 g of iodine in 160 ml of methanol. The mixture was stirred at 40° C and a total of 230 ml 4% KOH was added drop by drop over 20 min. The solution was then brought to pH 7.0 with 0.25 M formic acid and loaded onto a column (3 × 40 cm) of BioRad AG-50W x 8 (20—50 mesh), H^+ cycle, to remove cations. Elution was with 600 ml of 0.25 M formic acid. L-rhamnono-γ-lactone was located in the column eluate by the method of Hestrin [10] and recovered by lyophilization. The dry residue was taken up in 15 ml of water, loaded onto a column (3 × 40 cm) of BioRad AG-2 x 4 (200—400 mesh) formate cycle; the column was eluted with 1 l of 0.25 M formic acid and the L-rhamnono-γ-lactone-containing fractions were concentrated under reduced pressure to a light syrup. The syrup was triturated at 4° C with a mixture of 50 ml methanol, 40 ml diethyl ether, and 10 ml chloroform and stored in the cold for 24 h. The crystalline L-rhamnono-γ-lactone so obtained was removed by filtration and dried in vacuum. Yield = 2.0 g; m.p. = 149.5° C. Inffrared spectroscopy of the compound in a KBr disc showed a carbonyl absorption band at 1777 cm^{-1}. All other materials were reagent grade commercial products, unless otherwise stated. The organism used was a strain of *P. pullulans* obtained from Dr. S. Joly of the University of Sao Paulo. It was maintained on the growth medium solidified with 2% agar (Difco).

Paper chromatography

Ascending paper chromatography was performed on Whatman No. 1 filter paper in 1-propanol/formic acid/water (6 : 3 : 1, v/v/v) or 1-butanol/pyridine/water (6 : 4 : 3, v/v/v). Paper electrophoresis was carried out in 0.2 M ammonium acetate buffer, pH 5.8, on Whatman No. 1 paper at 24 V/cm.

Lactones were detected on paper as the hydroxamic acids [11]. Aldonic acids were detected by the same method after in situ lactonization accomplished by spraying with 0.2 M HCl and heating at 100° C for 15 min. Aldonic acids were also detected by spraying with 0.04% bromophenol blue in ethanol at pH 7.0 [12]. Th silver nitrate reagent of Trevelyan et al. [13] was used to visualize both lactones and aldonic acids.

Manometric studies

Manometric studies were carried out in the conventional Warburg apparatus. A 24-h culture of D-glucose or L-rhamnose-grown cells was washed twice with 0.15 M KCl and suspended in 0.1 M sodium phosphate (pH 5.6) to yield a suspension with a Klett reading of 530 (filter 42). A 0.2 ml amount of 0.1 M substrate (either D-glucose or L-rhamnose) was placed in the side arm; 2.5 ml of the cell suspension (containing 3.3 mg of cells, dry weight) was placed in the

main compartment of a Warburg flask, and 0.15 ml of 15% NaOH was placed in the center well. The experiment was started by mixing the substrate with the cell suspension; all measurements were done at 28° C with air as the gas phase.

Protein concentration

Protein was usually estimated by the method of Warburg and Christian [15]; in crude extracts and other preparations high in nucleic acid content, the method of Lowry et al. [16] was used, with crystalline bovine serum albumin as standard.

Carbohydrate assays

Reducing sugars were determined by the Somogy-Nelson method [14]. Aldonic acids were determined following conversion to the corresponding lactones by boiling in 0.2 M HCl for 5 min. Lactones were determined as hydroxamic acids by the method of Hestrin [10]. Pyruvate was determined by the method of Friedemann and Haugen [17].

Enzyme assays

L-Rhamnose dehydrogenase activity was measured by following the rate of reduction of NAD at 340 nm in the Beckman model DB spectrophotometer. The reaction mixture contained 0.3 ml of 50 mM Tris · HCl buffer (pH 9.0, 1 mM in EDTA), 0.05 ml of 10 mM NAD, 0.05 ml of 0.1 M L-rhamnose, and sufficient enzyme to produce an absorbance change of 0.01—0.03 min^{-1} in a total volume of 1 ml. The assay was carried out at 28° C in spectrophotometer cells with a light path of 1 cm. Under the assay conditions, the rate was constant with time and proportional to enzyme concentration. One unit of L-rhamnose dehydrogenase is defined as the amount of enzyme required to reduce 1 μmol of NAD per min in the standard assay.

To assay lactonase activity, reaction mixtures (0.3 ml) containing 10 μmol of L-rhamnono-γ-lactone, 40 μmol of Tris · HCl buffer (pH 7.0) and enzyme were incubated at 30° C and quenched at various times by the addition of 1.0 ml of hydroxylamine/NaOH reagent [10]. The absorbance was measured at 540 nm. A standard curve was constructed using a solution freshly prepared from authentic crystalline L-rhamnono-γ-lactone; controls to correct for nonenzymatic hydrolysis (less than 0.002 μmol/min) were included. Under the assay conditions used, the relation between protein concentration and reaction rate was linear. One unit of lactonase is defined as the amount of enzyme necessary to catalyze the hydrolysis of 1 μmol of substrate/min at 30° C.

L-Rhamnonate dehydratase, which catalyzes the conversion of L-rhamnonate to 2-keto-3-deoxy-L-rhamnonate, was detected by incubating the substrate with the enzyme preparation and testing for 2-keto-3-deoxy compounds by the semicarbazone method of McGee and Doudoroff [18], as well as by the periodate thiobarbiturate technique of Weissbach and Hurwitz [19].

Growth of cells

P. pullulans was grown in a liquid medium containing (per l): $(NH_4)_2SO_4$, 2 g; NH_4NO_3, 1 g; KH_2PO_4, 1.25 g; $Na_2HPO_4 \cdot 7H_2O$, 0.35 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $Fe(NO_3)_3 \cdot 7H_2O$, 14.5 mg; $ZnSO_4 \cdot 7H_2O$, 8.8 mg; $MnSO_4 \cdot 4H_2O$, 4 mg;

KCl, 0.75 g; NaCl, 0.25 g; $(NH_4)_6\ Mo_7O_4 \cdot 4H_2O$, 0.06 mg; $CaCl_2$, 0.1 g; $CuCl_2$, 3 mg, and yeast extract (Difco), 1 g. L-rhamnose or D-glucose (5 g) was autoclaved separately in 100 ml water and added to the sterile salts solution to yield 1 l of medium. Starter cultures were prepared by inoculating, from slants, 100 ml of medium contained in 500 ml Erlenmeyer flasks. Incubation was at 28° C on a rotary shaker at 130 rev./min. After a suitable time (usually 24 h) when the cultures attained a Klet reading of 200 units (filter No. 42), 10-ml portions were used to inoculate 500 ml of medium contained in 2-l Erlenmeyer flasks. Incubation was allowed to continue for 24 h at 28° C with shaking as described above, the cells were harvested by centrifugation at 3000 × *g* for 30 min and washed twice by suspension in 0.15 M KCl and recentrifugation. Yields of 5 g (wet weight)/l were obtained. Cells were stored at —15° C until used.

Preparation of cell-free extracts

This and all subsequent operations were done at 0—4° C. Cell-free extracts were prepared by grinding 15 g of packed cells in a chilled mortar with 15 g of glass beads (type 090-5005, Minnesota Mining and Mineral Corp., Minneapolis, Minn.) and 30 ml 50 mM Tris · HCl buffer, pH 9.0, 1 mM in respect to EDTA. The thick suspension was centrifuged for 30 min at 17 000 × *g*; the supernatant fluid was retained and the precipitate was suspended in 20 ml of the same buffer and again centrifuged. The two supernatant solutions were combined (crude extract).

Enzyme purification

(In $(NH_4)_2SO_4$ precipitations, 20 min elapsed between addition of $(NH_4)_2SO_4$ and centrifugation. The precipitates were centrifuged at 3000 × *g*.) Crude extract (40 ml) was mixed with 3.7 ml of 0.1 M $MnCl_2$. After 15 min the precipitate was removed by centrifugation at 20 000 × *g* and discarded ($MnCl_2$ supernatant). To the supernatant fluid was added a saturated solution of $(NH_4)_2SO_4$, pH 7.0, (18 ml) to 30% saturation. The precipitate was discarded, the supernatant fluid (58 ml) was brought to 60% saturation with 58 ml of saturated $(NH_4)_2SO_4$. The precipitate was collected and dissolved in 7.0 ml of 0.05 M Tris · HCl, pH 9.0, 0.1 mM in respect to EDTA ($(NH_4)_2SO_4$ fraction), and the solution was loaded onto a column (1 × 34 cm) of Sephadex G-50 equilibrated with 0.05 M Tris · HCl buffer, pH 9.0. The column was eluted with the same buffer and 2.5 ml fractions were collected. Fractions containing the highest activity were pooled (Sephadex G-50 filtrate). The Sephadex filtrate was applied to a column (1.6 × 40 cm) of DEAE cellulose equilibrated with 0.05 M Tris · HCl buffer, pH 9.0. The column was eluted with a linear pH and ionic strength gradient obtained by using a reservoir containing 200 ml of 0.5 M Tris · HCl, pH 7.0, and a mixing vessel containing 200 ml of 0.05 M Tris · HCl, pH 9.0. Flow rate was 18 ml/h and fractions of approximately 3.0 ml were collected. The major portion of the dehydrogenase activity emerged from the column in fractions 60—64 inclusive. These fractions were pooled to yield the purified enzyme (DEAE cellulose fraction). The most purified enzyme was present in fraction 61.

Results

Growth substrates. P. pullulans grew equally well on medium containing D-glucose or L-rhamnose as carbon and energy source with a generation time of 6 h at 28°C. Cell yield after 24-h growth was 5 g (wet weight)/l of medium.

Manometric studies. Cells grown on D-glucose-containing medium oxidized D-glucose with no lag but oxidized L-rhamnose only after a lag period of 1.5 h. L-Rhamnose grown cells oxidized either D-glucose or L-rhamnose immediately; each substrate was utilized at essentially the same rate. No L-rhamnose dehydrogenase activity was present in crude extracts of D-glucose-grown cells. Crude extracts of L-rhamnose-grown cells contained demonstrable L-rhamnose dehydrogenase (0.02 units/mg protein) as well as L-rhamnono-γ-lactonase activity (0.2 units/mg protein), and L-rhamnonate dehydratase.

Enzyme purification. As can be seen in Table I, an overall purification of 100-fold was achieved with 1.4% recovery of the dehydrogenase activity of crude extract. The purified L-rhamnose dehydrogenase was free of NADH oxidase, L-rhamnono-γ-lactonase, and L-rhamnonic acid dehydratase as well as of L-rhamnose isomerase [1] and L-rhamnulokinase [2].

Enzyme stability. Crude extract retains most of its activity for at least 6 months at −20°C, while the DEAE cellulose fraction loses 50% of its activity upon storage at 4°C for 1 week.

Optimal pH. The optimal pH of L-rhamnose dehydrogenase (determined using standard assay conditions with the exception that the pH was varied from 6 to 11) is 9.0 in either Tris · HCl or glycine/NaOH buffer. The activity decreases symmetrically on either side of this value; it is one-half the maximal value at pH 7.0 and 11.0.

Effect of substrate concentration. Apparent K_m values for L-rhamnose and NAD determined at pH 9.0 by the method of Lineweaver and Burk [20] were 0.2 mM and 0.02 mM, respectively. In these determinations the concentration of the non-varied substrate was identical to that used in the standard assay.

Nucleotide specificity. NADP could not substitute for NAD; no activity was observed when NADP was included in the standard assay mixture even at concentrations of 20 mM.

TABLE I

PURIFICATION OF L-RHAMNOSE DEHYDROGENASE

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
Crude extract	40	1400	28.0	0.02	—	—
$MnCl_2$ supernatant	42	1010	29.4	0.03	1.5	105
$(NH_4)_2SO_4$	7.2	360	27.7	0.08	4.0	99
Sephadex G-50 filtrate	11.8	194	15.3	0.08	4.0	55
DEAE-cellulose fractions 60—64	15	1.78	2.6	1.5	75.0	9
DEAE-cellulose fraction 61	2.5	0.2	0.4	2.0	100.0	1.4

TABLE II

EFFECT OF VARIOUS REAGENTS ON L-RHAMNOSE DEHYDROGENASE ACTIVITY

The enzyme was the DEAE-cellulose fraction with the highest specific activity. The incubation mixture described for the standard assay procedure was employed. EDTA, *p*-mercuribenzoic acid, sodium iodoacetate and various metal ions were added at the concentrations indicated.

Reagent	Concentration (mM)	Relative activity
None	—	100
$MgCl_2$	6.7	100
$CoSO_4$	6.7	50
$ZnCl_2$	6.7	25
$CuSO_4$	6.7	20
EDTA	20.0	63
p-Mercuribenzoate	2.0	10
Sodium iodoacetate	2.0	60

Substrate specificity. The following compounds (5 mM) were not substrates when used to replace L-rhamnose in the standard assay mixture: D- or L-arabinose, D- or L-fucose, D-galactose, D-glucose, D- or L-mannose, D- or L-xylose.

Activators and inhibitors. L-Rhamnose dehydrogenase showed no requirement for divalent cations; on the contrary, several divalent metal ions caused inhibition. Involvement of a sulhydryl group in enzyme activity was shown by the effect of *p*-mercuribenzoic acid, which inhibited 90% of enzyme activity at a concentration of 2 mM. The same concentration of iodoacetic acid inhibited 40% of the enzyme activity (Table II).

Reversibility and equilibrium. The oxidation of L-rhamnose to L-rhamnono-γ-lactone is a reversible reaction which could be demonstrated by measuring the oxidation of NADH in the presence of L-rhamnono-γ-lactone. Neither D-galactono-δ-lactone nor D-glucono-γ-lactone served as substrates for the enzyme or inhibited NADH oxidation in the presence of L-rhamnono-γ-lactone.

The reaction mixture (3 ml) contained 50 μmol of Tris/maleate (pH 6.8), 0.6 μmol NADH, 10 μmol of L-rhamnono-γ-lactone, and 0.64 unit of L-rhamnose dehydrogenase. At equilibrium the concentration of NADH was 0.126 mM. The equilibrium constant, K_{eq} for the reaction: L-rhamnose + $NAD^+ \leftrightharpoons$ L-rhamnono-γ-lactone + NADH + H^+, determined starting from either direction, was 12 μM.

Characterization of reaction products. The product of L-rhamnose dehydrogenase action in the forward direction was prepared by incubating 450 μmol of Tris/maleate buffer, pH 6.8; 300 μmol of L-rhamnose; 250 μmol of pyruvic acid (pH 6.5); 125 μg of lactic dehydrogenase; 5 μmol of NAD, and 1.4 units of L-rhamnose dehydrogenase in a total volume of 8.2 ml. After 2 h at 25°C, 150 μmol of lactone was formed and 100 μmol of pyruvate remained. The reaction mixture was deionized by passage through a column of Dowex 50 (200—400 mesh), H^+ cycle, the eluate was concentrated at 37°C and examined by paper chromatography in solvents 1 and 2. In addition to L-rhamnose, a substance with the chromatographic mobility of authentic L-rhamnono-γ-lactone was present in the reaction mixture.

The formation of L-rhamnose from L-rhamnono-γ-lactone by L-rhamnose dehydrogenase in the reverse direction was shown by paper chromatography in 1-propanol/formic acid/water (6 : 3 : 1, v/v/v) of the reaction mixture described in the reversibility experiment. At time zero the only detectable component was L-rhamnono-γ-lactone, which reacted with alkaline silver nitrate but gave no reaction when sprayed with *p*-anisidine · HCl (3% *p*-anisidine · HCl in 1-butanol/ethanol/water (4 : 1 : 1, v/v/v)) and held at 115° C for 10 min. At equilibrium, in addition to the lactone a compound with the mobility of L-rhamnose could be detected with the silver nitrate reagent; the *p*-anisidine spray, which is specific for reducing sugars, showed the presence in the mixture of a compound with the typical mobility and color reaction of authentic L-rhamnose.

Discussion

The soluble NAD-linked L-rhamnose dehydrogenase described in this paper is inducible since it is found only in L-rhamnose-grown cells. This is also consistent with the ability of *P. pullulans* grown on L-rhamnose, but not on D-glucose, to utilize L-rhamnose without lag. The enzyme is specific for L-rhamnose and oxidizes the furanose form of the sugar, as shown by the presence of L-rhamnono-γ-lactone as the sole reaction product. The reversibility of the dehydrogenation starting from the γ-lactone and the presence in extracts of the organism of a specific L-rhamnono-γ-lactonase are further evidence that L-rhamnono-γ-lactone is the product of the dehydrogenation.

Dahms and Anderson reported that extracts of a D-fucose-grown pseudomonad oxidized the substrate to D-fucono-δ-lactone [5]; the latter was enzymatically hydrolyzed to the free acid, which yielded 2-keto-3-deoxy D-fuconate upon dehydration [6]. This compound was cleaved by an aldolase to pyruvate and D-lactaldehyde [7].

Our preliminary results on the further metabolism of L-rhamnonate suggest that a similar pathway is operative in *P. pullulans* [8]. The evidence presented in this paper, and the work of Dahms and Anderson also make it seem likely that Eagon's so-called second pathway of L-fucose and L-rhamnose dissimulation [4] in bacteria involves the same sequence of reactions.

Acknowledgments

This investigation was supported in part by grants 9373 and 12741/69 from the National Research Council (CNPq), Brazil. D. S. F. was a Research Career Development Award (1-K3-GM 28,296) grantee of the National Institutes of Health, United States Public Health Service.

References

1 Takagi, Y. and Sawada, H. (1964) Biochim. Biophys. Acta 92, 10—17
2 Chiu, T.H. and Feingold, D.S. (1964) Biochim. Biophys. Acta 92, 489—497
3 Chiu, T.H. and Feingold, D.S. (1969) Biochemistry 8, 98—108
4 Eagon, R.G. (1961) J. Bacteriol. 82, 548—550
5 Dahms, A.S. and Anderson, R.L. (1972) J. Biol. Chem. 247, 2222—2227
6 Dahms, A.S. and Anderson, R.L. (1972) J. Biol. Chem. 247, 2228—2232
7 Dahms, A.S. and Anderson, R.L. (1972) J. Biol. Chem. 247, 2233—2237

8 Nakano, M., Rigo, L.U., Feingold, D.S. and Veiga, L.A. (1971) Proceedings of 5th Latin American Congress of Microbiology, Montevideo, Uruguay, p. 108
9 Moore, S. and Link, K.P. (1940) J. Biol. Chem. 133, 293—311
10 Hestrin, S. (1949) J. Biol. Chem. 180, 249—261
11 Abdel-Akher, M. and Smith, F. (1951) J. Am. Chem. Soc. 73, 5859—5860
12 Block, R.J., Durrum, E.L. and Zweig, G. (1958) in A Manual of Paper Chromatography and Electrophoresis, p. 128, Academic Press, New York
13 Trevelyan, W.E., Proctor, D.P. and Harrison, J.S. (1950) Nature 166, 444—445
14 Hestrin, S., Feingold, D.S. and Schramm, M. (1955) Methods Enzymol. 1, 234—245
15 Warburg, O. and Christian, H. (1941) Biochem. Z. 310, 384—421
16 Lowry, O., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Friedmann, T.E. and Haugen, G.E. (1943) J. Biol. Chem. 147, 415—442
18 MacGee, J. and Doudoroff, M. (1954) J. Biol. Chem. 210, 617—626
19 Weissbach, A. and Hurwitz, J. (1959) J. Biol. Chem. 234, 705—709
20 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658—666