Biochimica et Biophysica Acta, 613 (1980) 318—323

BBA 68998

Δ^1-PYRROLINE-5-CARBOXYLATE REDUCTASE FROM BAKER'S YEAST

PURIFICATION, PROPERTIES AND ITS APPLICATION IN THE ASSAYS OF L-Δ^1-PYRROLINE-5-CARBOXYLATE AND L-ORNITHINE IN TISSUE

TAKEO MATSUZAWA and ISAO ISHIGURO

Department of Biochemistry, School of Medicine, Fujita-Gakuen University, 1-98 Dengakugakubo Kutsukake-cho, Toyoake 470-11 (Japan)

(Received October 8th, 1979)

Key words: Δ^1-Pyrroline-5-carboxylate reductase; Pyrroline-5-carboxylate assay; Ornithine; (S. cerevisiae)

Summary

Δ^1-Pyrroline-5-carboxylate reductase (L-proline:NAD(P)$^+$ 5-oxidoreductase, EC 1.5.1.2) from Baker's yeast has been purified and characterized. Purification to an apparently homogenous protein was effected by using 'reagent-grade' water containing dithiothreitol and by maintaining a constant pH 7.5, because of the instability of the enzyme protein. The enzyme was purified approximately 200-fold from the crude extract of baker's yeast, and it is a negatively-charged protein with a molecular weight of 125 000, containing an active SH group which participates in binding with NAD(P)H. The K_m value for DL-pyrroline-5-carboxylate is $0.8 \cdot 10^{-4}$ M; for NADH, it is $4.8 \cdot 10^{-5}$ M; and for NADPH, it is $5.6 \cdot 10^{-5}$ M. These K_m values are much smaller than those of enzymes from other sources. The purified enzyme is free of contaminating enzymes which might interfere with its use in assays. The enzyme has been applied successfully to the assays of L-Δ^1-pyrroline-5-carboxylate and L-ornithine in tissue, and in vivo levels of these amino acids in rat liver are reported.

Introduction

Pyrroline-5-carboxylate reductase (L-proline:NAD(P)$^+$-5-oxidoreductase, EC 1.5.1.2) is widely distributed in vertebrates [1—5], microorganisms [4,6,7], and plants [8], and we have found that baker's yeast is also rich in this enzyme. In the course of a search for an enzyme with suitable properties to use as a coupling enzyme for the assay of L-ornithine, we found that the K_m values of the homogenous enzyme from Baker's yeast, against pyrroline-5-carboxylate

and NADH, were smaller than those of the partially purified enzymes from calf liver, rat liver and *Escherichia coli* reported previously [1,3,5,7].

In mammalian urea synthesis, the regulatory mechanism of the first process, carbamylphosphate synthetase I reaction by the acetylglutamate-arginine system, was discovered by Shigesada and Tatibana [9,10]. Several lines of investigation have also focused on the second process, the ornithine carbamyltransferase reaction, as the successive second site of the regulation, apparently in connection with the variation of the hepatic intramitochondrial ornithine level [10—14]. Therefore, an accurate and rapid method for the determination of the metabolites, pyrroline-5-carboxylate and ornithine, is essential to solve the problems in this field. This paper describes the purification of the enzyme from baker's yeast as an apparently homogenous protein. We have also examined its properties and its application to the assays of tissue levels of L-pyrroline-5-carboxylate and L-ornithine.

Materials and Methods

Chemicals and enzymes. DEAE-cellulose (DE-52) was obtainted from Whatman; NADH was from Oriental Yeast Industries (Tokyo); RNAase T_1, D-amino acid oxidase, and dithiothreitol were supplied by Boehringer Mannheim; D-proline was from P-L Biochemicals (Milwaukee, WI.); DL-pyrroline-5-carboxylate was chemically synthesized [15], but a better preparation has been recently reported and the precursor is commercially available [16]. 'Reagent-grade' water was prepared from distilled-deionized water by the Milli-Q system (Millipore); ornithine aminotransferase was purified as reported previously [17] and further purified on DEAE-Sephadex A-25 to remove contaminating enzyme activities. DL-Δ^1-pyrroline-2-carboxylate was prepared from D-proline by the action of D-amino acid oxidase [18], and was assayed by the method of Cabello et al. [19].

Enzyme assay. A Spectro-plus spectrophotometer (MSE Scientific Instruments, U.K.) was used for the measurements: the reaction mixture was placed in a 3 ml cell maintained at 37°C. The reaction mixture (total vol 3.0 ml) contained 100 μmol potassium phosphate buffer (pH 7.0), 0.33 μmol NADH, 0.5 μmol DL-pyrroline-5-carboxylate, and an appropriate amount of the enzyme solution. The enzyme was added using a Zymomixer (Fujihara Factory, Tokyo) to start the reaction, and the consumption of NADH was calculated from the decrease in absorbance at 340 nm, on the basis of the molar absorption coefficient of NADH ($6.27 \cdot 10^3$). One unit is defined as the activity consuming 1 μmol NADH per min at 37°C. Protein was measured by the method of Lowry et al. [20].

Purification. 'Reagent-grade' water containing $1 \cdot 10^{-4}$ M dithiothreitol was used for the preparation of all buffers and solutions.

Step 1. 500 ml cold acetone (—20°C) were added to 250 g dried yeast (Oriental Yeast Industries, Tokyo), blended for 1.5 min, filtered, washed with 500 ml acetone at —20°C, and dried. This procedure was repeated. The acetone-treated yeast was powdered in a mortar, suspended in 3 l 'reagent-grade' water (without dithiothreitol), and stirred at room temperature for 3 h.

The mixture was stirred further at 4°C overnight, centrifuged at 5000 × *g* for 10 min, and the supernatant collected.

Step 2. Potassium phosphate buffer (pH 7.5) was added to the crude extract to a final concentration of 0.01 M. 100 g DE-52, equilibrated with buffer, were added to the crude extract and the mixture was stirred for 30 min at 4°C. The slurry was filtered and the cellulose washed with 1 l 0.1 M potassium phosphate buffer (buffer 1). The enzyme was then eluted with 200 ml buffer 1 containing 0.3 M KCl, the elution was repeated twice and the eluates were combined.

Step 3. The eluate was concentrated with a 90 mM molecular filtration cell (Millipore) equipped with a PTGC membrane. The residue was dissolved in 15 ml 1 mM dithiothretiol/1 · 10^{-4} M EDTA; 20 μl (approx. 10 000 U) RNAase T_1 was added, and the mixture allowed to stand at room temperature for 1 h to digest RNA. Powdered $(NH_4)_2SO_4$ was added and the precipitate at 50—90% saturation, collected by centrifugation at 56 000 × *g* for 30 min. The precipitate was dissolved in a minimum volume of buffer 2 (0.01 M potassium phosphate buffer (pH 7.5)). These procedures (from the step 1 to the step 3) were carried out twice and the enzyme solutions combined. Unless otherwise noted, all further procedures were performed at 4°C.

Step 4. The enzyme solution from step 3 was applied to a Sepharose 6B column (2 × 38 cm) equilibrated and eluted with buffer 2. The active fractions were eluted just after the first minor peak and these fractions were collected.

Step 5 and 6. The enzyme solution was applied to a DEAE-Sephadex A-25 column (3 × 30 cm) equilibrated with buffer 2. The column was washed with approx. 100 ml 0.05 M potassium phosphate buffer (pH 7.5) and then eluted with a linear gradient of 0—0.25 M KCl in buffer 1 (250 ml buffer 1 + 250 ml 0.5 M KCl). The hydrostatic pressure was 55 cm H_2O. Fractions containing the enzyme activity eluted in a broad range between two peaks of contaminating proteins. The active fractions were collected, concentrated, and dialyzed overnight against buffer 2 containing 1 · 10^{-4} M EDTA. The dialyzed enzyme was applied to a small column (1.5 × 20 cm) of DEAE-Sephadex A-25 and eluted similarly as described above, but in half the volume to give a protein fraction containing the enzyme.

Measurement of molecular weight. Proteins ranging from 25 000 to 450 000 daltons were used as marker proteins. A Sepharose 6B column (2 × 38 cm) was equilibrated with 0.05 M potassium phosphate buffer (pH 7.5)/1 · 10^{-4} M dithiothreitol/0.1 M KCl and the elution volume (V_e) was measured from the absorbance peaks at 280 nm. V_e of the purified enzyme preparation was also determined by analysis of the enzyme activity.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out by the method of Ornstein and Davis [21]. 10 μl of the enzyme solution (approx. 20 μg protein) in 50% glycerol was applied to the gel.

Metabolite assay. The tissue was obtained from a decapitated rat and fixed as quickly as possible by the freeze-clamping method. 1—2 g of the frozen tissue was pulverized and homogenized in 5% $HClO_4$, then centrifuged at low speed. The supernatant was neutralized to pH 7.5 with KOH. Pyrroline-5-carboxylate and ornithine in the supernatant were determined as reported earlier [14]. The absorbance at 340 nm was determined with Beckman ultraviolet 5260 spectrophotometer.

Results and Discussion

Purification

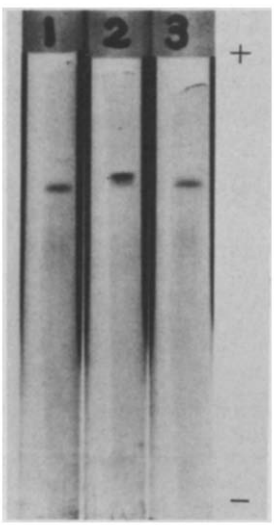
A typical purification from 1 kg dried yeast is shown in Table I. The enzyme was purified 187-fold and its recovery was approx. 20%, yielding 40 mg enzyme with a specific activity of 15 U per mg. The enzyme was free of pyridine nucleotide oxidase, alanine (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) and aspartate amino transferase (L-aspartate:2-oxoglurarate aminotransferase, EC 2.6.1.1), and lactate (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), malate (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) and glutamate dehydrogenase (L-glutamate:NAD^+ oxidoreductase (deaminating), EC 1.4.1.2) (each less than 0.5%).

The purified enzyme showed a single band in polyacrylamide gel electrophoresis and its mobility was very close to that of albumin, showing it to be negatively charged (Fig. 1).

Enzyme properties

The molecular weight of the enzyme was measured by gel chromatography with Sepharose 6B. The value of V_e for each protein was plotted as the logarithmic function of the molecular weight and the molecular weight of this enzyme was estimated to be 125 000.

The purified enzyme showed maximum absorbance at 278 nm, with a small



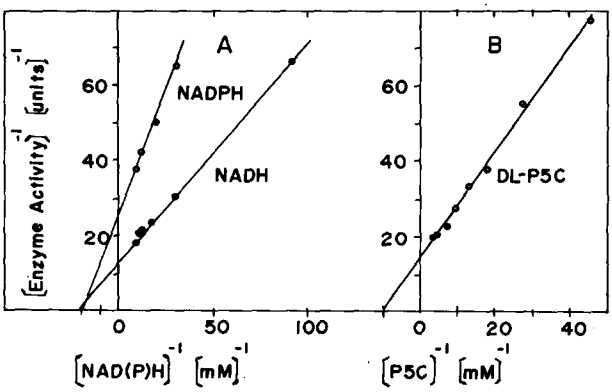


Fig. 1. Polyacrylamide gel electrophoresis of pyrroline-5-carboxylate reductase. The enzyme (20 μg, gels 1 and 3) and bovine serum albumin (gel 2) were subjected to polyacrylamide gel electrophoresis, and the gels were then stained for protein.

Fig. 2. Effect of NADH, NADPH, and DL-pyrroline-5-carboxylate on enzyme activity. A. The reaction mixture (3.0 ml) contained 100 μmol potassium phosphate buffer (pH 7.0), 0.5 μmol DL-pyrroline-5-carboxylate, a constant amount of the reductase, and NADH or NADPH at the indicated concentration. B. The reaction mixture (3.0 ml) contained 100 μmol potassium phosphate buffer (pH 7.0), 0.33 μmol NADH, a constant amount of the reductase and DL-pyrroline-5-carboxylate at the indicated concentration.

TABLE I

PURIFICATION OF BAKER'S YEAST PYRROLINE-5-CARBOXYLATE REDUCTASE

Step	Volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)
Crude extract	4528	38 500	3104	0.0803
DE 52	876	2 365	3914	1.637
Ammonium sulfate	30	590	1491	2.167
Sepharose 6B	32	288	1356	4.71
1st DEAE-Sephadex A25	22	110	864	7.86
2nd DEAE-Sephadex A25	28.5	39.9	597	15.0

shoulder at 290 nm. There was no absorption in the visible region. The ratio of A_{278} to A_{260} was 1.87.

The purified enzyme did not reduce acetaldehyde, formaldehyde, pyruvate, oxalacetate, or 2-oxoglutarate. DL-Pyrroline-2-carboxylate was not reduced by this enzyme. The enzyme was equally active with synthesized DL-pyrroline-5-carboxylate and its L-stereoisomer, formed from L-ornithine by the action of rat liver ornithine aminotransferase.

NADH, NADPH, and DL-pyrroline-5-carboxylate gave straight Lineweaver-Burk plots (Fig. 2). K_m values for NADH and NADPH were $4.8 \cdot 10^{-5}$ and $5.6 \cdot 10^{-5}$ M, respectively. V obtained by extrapolation of coenzyme concentration to infinity for NADPH was about a half of that for NADH, and K_m for DL-pyrroline-5-carboxylate was $0.8 \cdot 10^{-4}$ M. These K_m values against NADH and the substrate were the smallest among those of enzymes partially purified from calf liver, rat liver and *E. coli* (for NADH $8.4 \cdot 10^{-4}$, $2.5 \cdot 10^{-4}$ and $2.3 \cdot 10^{-4}$ M; for DL-pyrroline-5-carboxylate $3.3 \cdot 10^{-4}$, $2.0 \cdot 10^{-4}$ and $1.5 \cdot 10^{-4}$ M, respectively) [1,3,5,7].

The pH vs activity profile of this enzyme was over a broad range with optimum at pH 6—6.5.

The approximate value of the equilibrium constant, calculated when the

TABLE II

PYRROLINE-5-CARBOXYLATE AND ORNITHINE LEVELS IN RAT LIVER

Rats received a diet containing 5, 25 or 70% casein for 7 days each. The assay mixture contained 40 μmol potassium phosphate buffer (pH 7.5), 100 nmol NADH, 10 nmol dithiothreitol, 5 μmol 2-oxoglutarate, an appropriate amount of sample, and 0.25 unit of the reductase and ornithine aminotransferase each in a final vol of 1.0 ml. The absorbance at 340 nm without the enzymes was subtracted with those gained after each enzyme was added in turn and the reaction was run at 37°C for 30 min, and was also subtracted with the corresponding differences of the blank (minus sample). The amounts of two amino acids were calculated on the basis of the molar absorption coefficient of NADH ($6.27 \cdot 10^3$).

Protein content in diet	Number of samples	Ornithine	Pyrroline-5-carboxylate
		(nmol per g liver, Mean ± S.D.)	
5% casein	4	284.1 ± 81.5	—
25% casein	4	397.0 ± 54.0	27.0 ± 7.0
70% casein	4	765.1 ± 193.3	25.0 ± 15.0

enzyme reaction approached the state of equilibrium, was $1.2 \cdot 10^6$ at pH 7.0 indicating this enzyme is virtually a unidirectional enzyme as shown in rat tissues [5].

We found that the marked inactivation of enzyme activity during purification could be prevented when 'reagent-grade' water containing $1 \cdot 10^{-4}$ M dithiothreitol was used. Examination on the effect of various SH modifying reagents showed that the concentration resulting in 90% inhibition was $8 \cdot 10^{-6}$ M for *p*-hydroxymercuribenzoate, $2 \cdot 10^{-5}$ M for *N*-ethylmaleimide, and $1.2 \cdot 10^{-5}$ M for 5,5'-dithiobis(2-nitrobenzoic acid). Inhibition by iodoacetate was not marked. Inactivation by *N*-ethylmaleimide was prevented in the presence of NADH, but not with pyrroline-5-carboxylate. Activity inhibited by *p*-hydroxymercuribenzoate was recovered by the addition of excess dithiothreitol. These findings on the active SH group strongly suggest that it plays a crucial role in binding with NAD(P)H. This enzyme did not show the cold-sensitivity as reported for the rat liver enzyme [5]. The purified enzyme showed no marked inactivation for 6 months when dissolved in 50% glycerol containing $1 \cdot 10^{-4}$ M dithiothreitol and stored at −20°C.

Application to the assay of metabolites

Table II shows some findings on the pyrroline-5-carboxylate and ornithine levels in rat liver. The values obtained by this method showed a good agreement with those obtained by amino acid analysis (correlation coefficient, 0.97). This method was also strictly specific to these amino acids. The ornithine level seemed to adapt to dietary protein content, but the pyrroline-5-carboxylate was consistently very low without relation to dietary protein content, even when citrulline and ornithine accumulate markedly at an early phase after the transition from a 25% to a 70% casein diet [14].

References

1 Peisach, J. and Strecker, H.J. (1962) J. Biol. Chem. 237, 2255—2260
2 Adams, E. and Goldstone, A. (1960) J. Biol. Chem. 235, 3499—3503
3 Smith, M.E. and Greenberg, D.M. (1957) J. Biol. Chem. 226, 317—327
4 Meister, A., Radhakrishnan, A.N. and Buckley, S.D. (1957) J. Biol. Chem. 229, 789—800
5 Herzfeld, A., Mezl, V.A. and Knox, W.E. (1977) Biochem. J. 166, 95—103
6 Yura, T. and Vogel, H.J. (1959) J. Biol. Chem. 234, 335—338
7 Rossi, J.T., Vender, J., Berg, C.M. and Coleman, W.H. (1977) J. Bacteriol. 129, 108—114
8 Noguchi, M., Koiwai, A. and Tamaki, E. (1966) Agri. Biol. Chem. 30, 452—456
9 Shigesada, K. and Tatibana, M. (1971) J. Biol. Chem. 246, 5588—5595
10 Tatibana, M. and Shigesada, K. (1976) in The Urea Cycle (Grisolia, S., Bàgena, R. and Mayor, F., eds.), pp. 301—313, John Wiley and Sons, New York
11 Krebs, H.A., Hems, R. and Lund, P. (1973) Adv. Enzyme Regul. 11, 361—377
12 Katunuma, N., Okada, M. and Nishii, Y. (1966) Adv. Enzyme Regul. 4, 317—335
13 Saheki, T., Katsunuma, T. and Sase, M. (1977) J. Biochem. 82, 551—558
14 Matsuzawa, T. (1979) J. Nutr. Sci. Vitaminol. 25, 123—126
15 Stecker, H.J. (1971) Methods Enzymol. 17, 254, Academic Press, New York
16 Mezl, V.A. and Knox, W.E. (1976) Anal. Biochem. 74, 430—440
17 Matsuzawa, T., Katsunuma, T. and Katunuma, N. (1968) Biochem. Biophys. Res. Commun. 32, 161—166
18 Krebs, H.A. (1939) Enzymologia 7, 53—57
19 Cabello, J., Leon, B., Projoux, V. and Plaza, M. (1964) Arch. Biochem. Biophys. 107, 51—56
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
21 Ornstein, L. and Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 321—349