Pyrroline-5-carboxylate Reductase of *Neurospora crassa*: Partial Purification and Some Properties*

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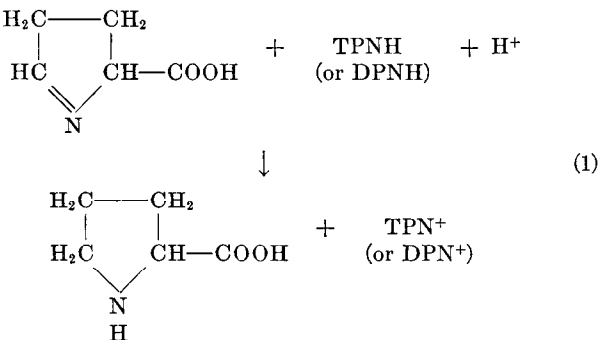
Previous studies (1, 2) with mutant and wild type strains of *Neurospora crassa* have pointed to glutamic γ-semialdehyde and its spontaneously cyclized form, Δ^1-pyrroline-5-carboxylate, as intermediates in the biosynthesis of proline. These studies, based largely on experiments *in vivo*, have recently been substantiated through work *in vitro* with extracted enzymes (3, 4). The terminal step in proline formation, namely the reduction of PC,[1] was shown (3) to be catalyzed by pyrroline-5-carboxylate reductase according to the following schematic equation:



(1)

The present paper is concerned with a characterization of this enzyme.

EXPERIMENTAL

Materials

Organisms—The organisms used are a wild type strain (St. Lawrence 74A) and a proline-requiring mutant strain (21863 (5)) of *N. crassa*.

Compounds—The substrate, PC, was synthesized as previously described (6). L-Proline was obtained from General Biochemicals, Inc. TPNH and DPNH were products of the Sigma Chemical Company.

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[1] The abbreviation used is: PC, Δ^1-pyrroline-5-carboxylate.

Assay of Enzyme Activity

Standard Assay Conditions—The activity of the enzyme was assayed through the measurement of the optical density decrease (at 340 mμ) resulting from the oxidation of TPNH or DPNH. Assays were run at 25° in a Beckman spectrophotometer (light path, 1 cm.). The complete system contained 300 μmoles of potassium phosphate at pH 7, 6 μmoles of PC, 0.6 μmole of TPNH or DPNH, enzyme solution, and water to 3 ml. A control mixture, in which PC was omitted from the complete system, was prepared for each assay. The reaction was usually started by the addition of enzyme, and optical density readings were taken every 1 or 2 minutes for 10 to 20 minutes. Since the reaction was found to follow first order kinetics in which the pyridine nucleotide is rate-limiting, reaction rates are expressed in terms of the first order velocity constant, k.

Unit and Specific Activity of Enzyme—A unit of pyrroline-5-carboxylate reductase is defined as the amount of enzyme that will give $k = 1 \times 10^{-3}$ minutes^{-1} under standard conditions when DPNH is used as nucleotide. The specific activity of a given reductase preparation is taken as units of the enzyme per mg. of protein in this preparation.

Protein Determination—Protein was determined by the method of Lowry *et al.* (7).

Partial Purification of Enzyme

Crude Enzyme Extract—For the preparation of crude extracts, mycelia (1.0 part, wet weight) of *N. crassa* grown on minimal medium (8) at 30° were harvested, rinsed with distilled water, squeezed, and extracted by grinding with sand (0.5 part) in cold 0.1 M potassium phosphate (0.5 part) at pH 7. The resulting mixture was centrifuged at about 100,000 × g for 1 hour at approximately 3°, and the supernatant fluid was used as crude extract (enzyme Preparation A). Crude extracts could also be prepared from lyophilized mycelia. The subsequent steps were carried out at 0–5°.

Protamine Treatment—Protamine sulfate, dissolved in 0.1 M potassium phosphate buffer (pH 7) to give a 1.5 per cent solution (pH 6.7), was added to the crude extract in the proportion of 1 ml. of protamine solution per 100 mg. of protein in the extract. After 15 minutes, the resulting mixture was clarified by centrifugation (Fraction B), and the sediment obtained was discarded.

First Ammonium Sulfate Fractionation—To Fraction B, powdered ammonium sulfate was added to 25 per cent of saturation. The saturating amount (at approximately 0°) of am-

monium sulfate was taken as 720 gm. added to 1 l. of aqueous solution. The precipitate thus obtained was removed by centrifugation and discarded. The resulting supernatant solution was brought to 35 per cent of saturation and the precipitate formed was collected and dissolved in 0.1 M potassium phosphate at pH 7 (Fraction C).

Ammonium Phosphate Fractionation—Fraction C was further fractionated by the addition of powdered dibasic ammonium phosphate. For this salt, the saturating amount was taken as 430 gm. added to 1 l. of aqueous solution. The fraction obtained between 35 and 45 per cent of saturation was collected and dissolved in 0.1 M potassium phosphate at pH 7 (Fraction D).

Second Ammonium Sulfate Fractionation—Fraction D was then fractionated with ammonium sulfate. The fraction between 26 and 32 per cent of saturation was collected, dissolved in 0.1 M potassium phosphate at pH 7, and dialyzed against a solution of 1 mM glutathione in the same buffer (Fraction E). Glutathione served as a stabilizer of the enzyme.

A summary of the purification procedure in a typical experiment is presented in Table I. Enzyme assays were performed after each enzyme preparation had been dialyzed against 0.1 M potassium phosphate (pH 7) containing 1 mM glutathione.

TABLE I

Summary of enzyme purification

Enzyme fraction	Total volume	Protein	Enzyme activity	Specific activity	Overall yield
	ml.	*mg.*	*units*	*units/mg. protein*	%
A. Crude extract	200	2500	45,000	18	100
B. Protamine-treated	222	1600	43,200	27	96
C. First ammonium sulfate	20	260	36,700	141	82
D. Ammonium phosphate	5	75	28,800	385	64
E. Second ammonium sulfate	3	32	19,700	615	44

TABLE II

Effect of pH on PC reductase activity

The conditions employed were standard, except that the pH was varied by the use of suitable mixtures of KH_2PO_4 and K_2HPO_4 at constant total phosphate concentration (0.1 M). The final pH was determined with a Beckman model G pH meter.

pH	Relative reaction rate	
	TPNH	DPNH
5.5	50	89
6.0	72	100
6.5	87	86
7.0	100	67
7.5	94	51
8.0	78	39

TABLE III

Specific activity of PC reductase at various growth stages

Mycelia of the organism were grown at 30° in Fernbach flasks containing 400 ml.of Fries minimal medium with or without 0.01 per cent L-proline; they were harvested, lyophilized, and extracted with cold 0.1 M potassium phosphate buffer. The resulting extracts were used for the specific activity determinations.

Period of incubation	Specific activity		Repression
	Growth without proline	Growth with proline	
days			%
1.3	28.3	20.7	27
1.9	22.1	14.3	35
3.0	13.0	6.7	48
4.0	12.8	6.9	46
5.0	13.1	7.1	46
6.0	16.8	13.3	21
7.0	14.0	12.9	8
9.0	19.0	17.4	9

Properties of Enzyme and of Reaction

Pyridine Nucleotide Requirement—The reduction of PC depends on the presence of reduced pyridine nucleotide in the enzymatic reaction mixtures. Either TPNH or DPNH is required; the activity with TPNH is about 16 times that with DPNH under standard conditions. This ratio did not change appreciably when the enzyme was purified over 40-fold, or when Fraction C was 90 per cent inactivated through dialysis and storage in the absence of glutathione. Thus, it appears that a single enzyme is involved in the reactions with either of the pyridine nucleotides. No reduction of PC occurred when boiled enzyme was used.

Reduction Product—The product of the enzymatic reduction of PC was found to be proline by paper chromatography, by bioautography with proline-requiring mutant strain 55-1 (6) of *Escherichia coli*, and by the characteristic yellow color obtained through reaction with ninhydrin. It was further shown that for each mole of either TPNH or DPNH oxidized (as determined spectrophotometrically), approximately 1 mole of L-proline (as determined by bioassay) was produced.

Effect of Concentration of PC on Reaction—The effect of concentration of PC on the reaction velocity was examined over a range of initial concentrations from 1 to 6 μmoles of this compound per 3 ml. under otherwise standard conditions. The concentrations of PC (on the basis of the L form) giving half-maximal velocity were calculated (9) to be $K_m = 4.5 \times 10^{-4}$ M for the reaction with TPNH and $K_m = 5.3 \times 10^{-4}$ M for the reaction with DPNH. It should be pointed out that these values are based on PC concentrations determined by bioassay which gives only approximations (6).

Effect of pH on Reaction—The optimal pH for the reaction was found to be about 7.0 with TPNH and about 6.0 with DPNH, as shown in Table II.

Effect of Cultivation Conditions on Specific Enzyme Activity—Crude enzyme Preparations A were made from lyophilized mycelia grown in minimal medium and harvested at various stages of growth, and the specific activity of these preparations was determined. It can be seen from Table III that the specific activity is relatively high in the early growth phases and then declines rapidly. In early stationary phase (3rd to 5th day), the specific activity reaches a minimum and subsequently increases to some extent.

It is also apparent from Table III that the specific activity of the reductase decreases markedly when mycelium is grown in minimal medium supplemented with proline (100 μg. per ml.)

rather than in unsupplemented minimal medium. This decrease of specific activity, apparently reflecting repression of enzyme formation (*cf.* (10)), is seen to vary as a function of the age of the culture. The greatest repression, amounting to nearly 50 per cent, was observed on the 3rd day of cultivation.

Reductase Activity of Extracts of Proline-requiring Mutant—A previous study (1) has provided nutritional and other evidence that proline-requiring mutant strain 21863 may be blocked in the conversion of PC to proline. In agreement with expectations, it was found that extracts of this strain had greatly diminished reductase activity. The level of reductase activity in one of the reisolates of this strain, 21863-6A, was about 0.2 per cent of that of the wild type strain used.

Crude extracts were prepared from the mutant mycelium in essentially the same way as described above for the wild type, except that the mutant was grown in the presence of proline (100 μg. per ml.) and the resulting mycelium was lyophilized, powdered, and then extracted by shaking in buffer. Assay of enzyme activity in mutant extracts was carried out either by determination of proline formed (using strain 55-1 of *E. coli* as assay organism) in the case of crude extracts, or by oxidation of TPNH in the case of partially purified extracts.

In order to test for possible interactions between reductase preparations from the mutant and from the wild type strains, appropriate mixture experiments were performed. As shown in Table IV, the reductase activities from the two sources are additive. These experiments thus give no indication of the presence of any inhibitors in the mutant extracts or of any stimulators in the wild type extracts.

Comparisons of some of the properties of the mutant reductase with those of the wild type enzyme have been made and the results of these studies will be the subject of another communication.

TABLE IV

Mixture experiment with PC reductase from mutant and wild type strains

The complete assay mixtures contained 100 μmoles of potassium phosphate at pH 7, 9 μmoles of PC, 1.5 μmoles of TPNH, enzyme solution, and water to 1.2 ml. After incubation for 2 hours at 25°, the reaction was stopped and the proline produced was determined by bioassay with strain 55-1 of *E. coli*. Control experiments in which PC was omitted were run for each assay. The enzyme preparations employed were the first ammonium sulfate fraction of reductase from mutant strain 21863-6A or from wild type strain 74A or a mixture of these two fractions.

Enzyme preparation	Amount of enzyme	Amount of protein	L-Proline formed
	units	*mg.*	*μmoles*
Mutant	0.53	10.2	0.14
Mutant	1.05	20.4	0.23
Wild	0.60	0.005	0.17
Mutant + wild	0.53 + 0.60	10.2 + 0.005	0.26*

* Value expected for additivity, 0.25 μmole (by extrapolation)

DISCUSSION

The present study has substantiated the supposed roles of glutamic γ-semialdehyde and PC as intermediates in proline formation in *N. crassa*. That pyrroline-5-carboxylate reductase actually has the expected biosynthetic function is indicated by the finding that a mutant strain, considered on nutritional grounds to be blocked in the last step of proline formation, showed sharply diminished reductase activity compared to that of the wild type. Moreover, genetic and enzymatic studies on "leakage" with several reisolates of this mutant led to the conclusion that in *N. crassa*, at least under the conditions that prevailed, there probably is no major pathway leading to proline, except the one via the reduction of PC (11). Interestingly enough, an enzyme catalyzing the reduction of Δ^1-pyrroline-2-carboxylic acid to form L-proline has recently been extracted from both wild type strain 74A and mutant strain 21863 of *N. crassa* (12). It was also found that Δ^1-pyrroline-2-carboxylic acid could partially satisfy the proline requirement of strain 21863 (12). The existence of a minor or contributory pathway to proline is thus indicated.

After a preliminary announcement (3) of the present findings, a similar enzyme was discovered in extracts of rat liver (13, 14), *E. coli*,[2] and *Aerobacter aerogenes* (12). It thus appears that the mode of proline formation demonstrated in *N. crassa* is not confined to this organism. It is noteworthy that the pyrroline-5-carboxylate reductases of *N. crassa* and *E. coli* are more active with TPNH than they are with DPNH, but the opposite is true for the corresponding enzyme from rat liver.

SUMMARY

Pyrroline-5-carboxylate reductase has been extracted from *Neurospora crassa* and partially purified by a procedure including treatment with protamine and fractionation with ammonium sulfate and ammonium phosphate.

Under the conditions used, the substrate (Δ^1-pyrroline-5-carboxylate) concentration giving half-maximal velocity is approximately 4.5×10^{-4} M for the reaction with TPNH and 5.3×10^{-4} M for the reaction with DPNH (on the basis of L-Δ^1-pyrroline-5-carboxylate). The optimal pH is about 7.0 for the TPNH reaction and about 6.0 for the DPNH reaction.

The specific activity of the enzyme was found to vary with the growth stage of the culture and was also found to be decreased by the addition of L-proline to the culture medium.

Extracts of a proline-requiring mutant of *N. crassa* showed greatly diminished reductase activity (0.2 per cent of that of the wild type).

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