Studies on Nitrogen Metabolism in Tobacco Plants

Part VII. Δ^1 -Pyrroline-5-carboxylate Reductase from Tobacco Leaves

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Δ^1 -Pyrroline-5-carboxylate reductase has been considerably purified from tobacco leaves. This enzyme uses NADPH or NADH for the formation of proline, although the former is better used. This enzyme was found in washed chloroplast extract as well as in cytoplasmic fluid and utilized NADPH, formed by the photosynthetic NADP reduction, for the synthesis of proline in the light.

As previously reported,¹⁾ the biosynthesis of proline from glutamate in tobacco leaves is highly active in the daytime whereas it proceeds very slowly at night, although the oxidation of proline to glutamate takes place at a comparatively slow rate in the daytime and at night without any appreciable difference. These results suggested that light might play an important role in the biosynthesis of proline from glutamate in green leaves, possibly by means of photoreduction and photophosphorylation.

Although proline has been shown to be synthesized in various microorganisms from glutamate through glutamic- γ -semialdehyde and its cyclization product, Δ^1 -pyrroline-5-carboxylate,^{2,3)} no study is yet available on the enzymes of this pathway in higher plants. In order to elucidate the role of light in the biogenesis of proline in green leaves, these enzyme systems in tobacco leaves have been investigated and this paper describes the occurrence of Δ^1 -pyrroline-5-carboxylate reductase in tobacco chloroplasts and a possible role of light in this reaction.

1) S. Mizusaki, M. Noguchi and E. Tamaki, *Arch. Biochem. Biophys.*, **105**, 599 (1964).

2) H. J. Vogel and B. D. Davis, *J. Am. Chem. Soc.*, **74**, 109 (1952).

3) H. J. Vogel and D. M. Bonner, *Proc. Natl. Acad. Sci.*, **40**, 688 (1954).

EXPERIMENTAL

Broken Chloroplasts and Chloroplast Extract.

Broken chloroplasts and chloroplast extract were prepared according to the method of Whatley, Allen and Arnon⁴⁾ for spinach chloroplasts with slight modification. Plants of *Nicotiana tabacum*, "Bright Yellow", were grown in a green house and young expanded leaves were harvested usually in the morning prior to each experiment. After the leaves had been washed with distilled water, midribs were removed from the leaves and discarded.

Leaf blades (25 g) were sliced into pieces and ground by hand in a precooled mortar with 50 ml of 0.35 M NaCl solution which contained both sodium ascorbate and mercaptoethanol at a concentration of 0.01 M, 5 ml of 0.5 M Tris-HCl buffer pH 8.0, and about 10 g of silica sand. The slurry was squeezed through four layers of cheesecloth, and the filtrate was centrifuged for 1 minute at 200 g. The supernatant fluid was further centrifuged for 7 minutes at 1,000 g, and the resultant sediment was suspended in 25 ml of washing medium consisting of 0.35 M NaCl, 0.01 M sodium ascorbate, and 0.01 M mercaptoethanol. The suspension was centrifuged for 7 minutes at 1,000 g, and the green sediment (washed chloroplasts) was carefully resuspended in 5 ml of ice-cold hypotonic solution consisting of 0.035 M NaCl, 0.01 M sodium ascorbate, and 0.01 M mercaptoethanol. The suspension was centrifuged for 10 minutes at 18,000 g. The supernatant liquid was used as chloroplast ex-

4) F. R. Whatley, M. B. Allen and D. I. Arnon, *Biochim. Biophys. Acta*, **32**, 32 (1959).

tract. The lower layer of the sediment consisted of strach grains which were brought out by the disruption of chloroplasts. The green upper layer was resuspended in 5 ml. of the hypotonic solution described above and used as broken chloroplasts. All these procedures were carried out at 0~4°C.

Tobacco Ferredoxin.

Tobacco ferredoxin was extracted and purified from young tobacco leaves according to the method of Tagawa and Arnon for spinach ferredoxin,⁵⁾ omitting the last step for the crystallization. Specific activity of the ferredoxin preparation was 38.9 units per mg protein, according to the definition by San Pietro and Lang.⁶⁾

Reactions under Illumination.

Photosynthetic reactions with chloroplast fragments were carried out aerobically in 50 ml Erlenmeyer flasks with a flat bottom. The flasks were immersed in a glass water bath kept at 20°C and illuminated from the bottom by a bank of 150-watt tungsten bulbs, approximately 20,000 lux at the level of the reaction flasks. The reaction was started by turning on the light and terminated after one hour by turning off the light and adding 0.3 ml of 2N HCl. The acidified reaction mixtures were heated in a boiling water bath for 1 minute, cooled immediately, and centrifuged. The whole supernatant liquid was concentrated in vacuo to dryness, and subjected to the assay of proline.

Assay of the Enzyme Activity.

Optical assay of the enzyme activity was carried out as follows. Ten micromoles of DL- Δ^1 -pyrroline-

5-carboxylate, 0.4 micromole of NADPH and enzyme were incubated at 25°C in 0.025 M phosphate buffer, pH 7.0, in a total volume of 3.0 ml. The decrease in absorbancy at 340 m μ for the initial 3 minutes was followed and correction was made for the endogenous oxidation of NADPH. An enzyme unit is defined, according to Peisach and Strecker,⁷⁾ as the enzyme amount which produces a decrease in optical density of 0.001 per minute at 340 m μ (1.0 cm light path).

Reagents and Analytical Procedures.

DL- Δ^1 -Pyrroline-5-carboxylate was prepared by the method of Strecker et al.^{7,8)} and neutralized with Na₂CO₃ before use. Chlorophyll was estimated by the method of Arnon⁹⁾ and protein by the method of Lowry et al.¹⁰⁾ Proline was determined with a Beckman/Spinco Amino Acid Analyzer. NADP, NADPH and NADH were obtained from Boehringer & Soene GmbH, Mannheim, Germany.

RESULTS AND DISCUSSION

Distribution of Δ^1 -Pyrroline-5-Carboxylate Reductase in Tobacco Leaves.

Enzymic reduction of Δ^1 -pyrroline-5-carboxylate (hereafter abbreviated to PCA) by various cell fractions of tobacco leaves are listed in Table I with glutamic acid decarboxylase activity of each fraction.

As shown in the table, the specific activity of PCA reduction with the chloroplast extract was similar to that with the supernatant. On

TABLE I. REDUCTION OF PCA AND DECARBOXYLATION OF GLUTAMATE BY CELL FRACTIONS

Cell fraction	μ mole proline produced ^{a)} per mg protein per hr.	μ mole glutamate decarboxylated ^{b)} per mg protein per hr.
200~1,000 g (washed chloroplasts)	0.288	
1,000~18,000 g (grana and mitochondria)	0.236	
18,000 g supernatant	1.25	4.90
chloroplast extract	1.68	0.82

a) The reaction mixture contained in a total volume of 2 ml, 2~10 mg protein of each fraction, 100 μ moles of phosphate buffer, pH 7.0, 5 μ moles of NADPH and 10 μ moles of DL-PCA. Reactions were carried out for 30 min. at 30°C in the dark. Control experiments were carried out without NADPH.

b) The glutamic acid decarboxylase was assayed by measuring the radioactivity of γ -aminobutyric acid formed from U-C¹⁴-glutamic acid using a Beckman/Spinco Amino Acid Analyzer and a Packard Tri-Carb Flow Monitor System. The reaction mixture contained in a total volume of 2 ml, 0.3 to 0.9 mg protein, 100 μ moles of phosphate buffer, pH 5.7, and 10 μ moles (1 μ C) of U-C¹⁴-glutamic acid. Reactions were carried out for 30 min. at 30°C.

5) K. Tagawa and D.I. Arnon, "Modern Method of Plant Analysis," Vol. VII, Springer-Verlag, 1964, p. 595.

6) A. San Pietro and H.M. Lang, *J. Biol. Chem.*, **231**, 211 (1958).

7) J. Peisach and H.J. Strecker, *J. Biol. Chem.*, **237**, 2255 (1962).

8) H.J. Strecker, *J. Biol. Chem.*, **235**, 2045 (1960).

9) D.I. Arnon, *Plant Physiol.*, **24**, 1 (1949).

10) O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

the other hand, glutamic acid decarboxylase activity in the supernatant is much higher than that in the chloroplast extract. As these results suggested that PCA reductase might be concerned with chloroplasts as well as with cytoplasm, chloroplast extracts were used as crude enzyme in this study.

PCA Reduction with Chloroplast Extract in the Dark.

Table II shows that PCA is reduced to proline in the dark with chloroplast extract in the presence of NADPH or NADH as a hydrogen donor. Both NADH and NADPH have been reported to serve as hydrogen donors for PCA reduction, although the activity with NADPH is much higher than that with NADH in *Neurospora*,¹¹⁾ whereas the reverse trend is observed with mammalian liver.^{7,12)}

TABLE II. REDUCTION OF PCA WITH CHLOROPLAST EXTRACT

	Content of proline (μ mole)
Expt. A	
complete	2.40
complete minus NADPH	0.10
complete minus PCA	0.11
complete minus chloroplast extract	0.13
Expt. B	
Hydrogen donor	
NADPH	2.78
NADH	2.32
none	0.04

Complete reaction mixture contained in a total volume of 3 ml; 1 ml of chloroplast extract, 80 μ moles of Tris buffer pH 7.5, 10 μ moles of DL-PCA, and 5 μ moles of reduced pyridine nucleotide. In Expt. A, NADPH was used as the hydrogen donor. Reactions were carried out for 1 hour at 30°C and stopped by adding 0.3 ml of N hydrochloric acid into the mixture.

In the case of chloroplast extract, NADPH seems to be a little better utilized than NADH although the difference is less significant than that in the purified enzyme described below.

11) T. Yura and H. J. Vogel, *J. Biol. Chem.*, **234**, 335 (1959).

12) M. E. Smith and D. M. Greenberg, *ibid.*, **226**, 317 (1957).

Purification of PCA Reductase in Tobacco Leaves.

Since PCA reductase in higher plants has not yet been studied, partial purification of this enzyme from tobacco leaves was undertaken.

Young expanded tobacco leaves (1 kg), freed from midribs and freezed in a deep-freezer, were macerated with 3l of 0.1M phosphate buffer, pH 7.5, which contained sodium ascorbate (0.01 M), and EDTA (0.002M), in a Waring blender for 5 minutes. The homogenate was filtered through four layers of cheesecloth and the residue was discarded. The filtrate was centrifuged at 2,500g for 15 minutes and the sediment was discarded. To the supernatant fluid was added a calculated amount of powdered ammonium sulfate to give 25% saturation; pH of the fluid was maintained between 7 to 7.5 with aqueous ammonia during the addition procedure. The precipitate thus obtained was removed by centrifugation at 16,000g for 5 minutes and discarded. The supernatant solution was further brought to 50% saturation with ammonium sulfate and the resulting precipitate, which had the majority of the enzyme activity, was collected by centrifugation and dissolved in 300ml of 0.05M phosphate buffer, pH 7.0, containing 0.005M mercaptoethanol and 0.001M EDTA* (Fraction A).

Fraction A was again fractionated with ammonium sulfate and the precipitate obtained between 30 and 45% saturation was dissolved in 150ml of the phosphate buffer (Fraction B). Fraction B was further treated with ammonium sulfate, and the precipitate obtained from 35 to 45% saturation was dissolved in 10ml of the phosphate buffer (Fraction C). Fraction C was then passed through a column of Sephadex G-200 (3cm $\times$ 25cm) equilibrated with the same phosphate buffer. The main PCA reductase fraction (Fraction D) thus obtained was fractionated

* This buffer solution was used to dissolve the enzyme throughout the purification procedure.

TABLE III. SUMMARY OF PURIFICATION PROCEDURES

Fraction	Total units	Total protein (mg)	Specific activity units/mg protein	Yield (%)
Crude supernatant fluid	555,000	41,700	13.3	100
Fraction A (Ammonium sulfate I)	792,000	12,700	62.4	143
Fraction B (Ammonium sulfate II)	606,000	2,640	230	109
Fraction C (Ammonium sulfate III)	508,000	1,500	338	91.5
Fraction D (Sephadex treatment I)	462,000	1,130	409	83.2
Fraction E (Ammonium sulfate IV)	325,000	511	636	58.6
Fraction F (Sephadex treatment II)	145,000	134	1,080	26.1
Fraction G (Ammonium sulfate V)	138,000	65.7	2,100	24.9

As for the assay procedure, see the text.

with ammonium sulfate at 45 to 50% saturation and the fraction was dissolved in 10 ml of the phosphate buffer (Fraction E). Fraction E was passed through a Sephadex G-200 column (3×25 cm) and the fractions which had high PCA reductase activity were collected (Fraction F). Fraction F was finally fractionated with ammonium sulfate, at 45 to 50% saturation and the precipitate obtained was dissolved in 10 ml of the phosphate buffer (Fraction G). All steps in the purification were carried out at 0 to 4°C.

A summary of the yields and purifications obtained at each step in the preparation of the enzyme is presented in Table III.

Treatment of the enzyme preparation with protamine sulfate, or chromatography with a DEAE-cellulose column resulted in a large loss of activity, although the purity of the enzyme was doubled by the latter procedure.

The enzyme is relatively stable in the solution with mercaptoethanol and can be stored at room temperature for a few days or at 4°C for more than 2 weeks with little or no loss in activity.

pH Optimum and Pyridine Nucleotide Specificity.

As shown in Fig. 1, the optimum pH for the enzyme action was observed around 7.0, either with NADH or NADPH as the hydrogen donor. However, the activity with NADPH was relatively higher than that with NADH within the tested pH range.

FIG. 1. pH Dependence and Pyridine Nucleotide Specificity of the Enzyme.

Assay procedures were as given in the text.

TABLE IV. INHIBITORS OF PCA REDUCTASE OF TOBACCO LEAVES

Component	Concentration (M)	Inhibition (%)
ATP	1.0×10^{-3}	46
	3.3×10^{-3}	63
ADP	1.0×10^{-3}	14
	3.3×10^{-3}	46
NADP	1.7×10^{-3}	54
NAD	1.7×10^{-3}	15
L-proline	1.0×10^{-3}	0
	1.0×10^{-2}	8
Mg ⁺⁺	3.3×10^{-3}	0
Ag ⁺	1.7×10^{-4}	52
NH ₄ ⁺	1.0×10^{-2}	7
p-chloromercuribenzoate	1.0×10^{-5}	25

Enzyme used was from the last step in purification, at the concentration of 4.3 µg per ml. Assay procedure was the same as described in the text.

Inhibitors.

PCA reductase in tobacco leaves seems to be an SH enzyme as the enzyme from other sources,^{7,11,12)} for it is inhibited by Ag^+ and *p*-chloromercuribenzoate as seen in Table IV. The enzyme is also inhibited by ATP, ADP, NAD and NADP. Proline does not inhibit the enzyme even in the concentration of 10^{-2} M, although some authors^{7,13)} have reported that proline inhibits the enzyme in relatively high concentrations.

Reduction of PCA to Proline with Chloroplasts under Illumination.

As mentioned above, the biosynthesis of proline in tobacco leaves is greatly enhanced in the light. This enhancement seems to be operative either in the step of the reduction of glutamate to PCA or in that of PCA to proline, or in both steps. In PCA reduction, which requires NADPH as a hydrogen donor, the effect of light on the reaction seems to be in the photoreduction of NADP to NADPH. In order to confirm this assumption, reduction of PCA to proline with NADP and chloroplasts under illumination was carried out. As

TABLE V. PCA REDUCTION UNDER ILLUMINATION

Treatment	Proline Content (μmole)
light, complete	2.24
light, minus chloroplast extract	0.18
light, minus PCA	0.11
light, minus NADP	0.10
dark, complete	0.19

The complete mixture contained, in a final volume of 3 ml; chloroplast extract, 1.5 ml (equivalent to 2.8 mg chlorophyll), broken chloroplasts containing 0.48 mg of chlorophyll, 3 units of purified tobacco ferredoxin, and the followings in μmoles : Tris, pH 7.5, 80; MgCl_2 , 5; MnCl_2 , 2; NADP, 1; DL-PCA, 10. As for the other reaction conditions, see the text.

shown in Table V, proline was produced from PCA with chloroplast extract and NADP in the presence of broken chloroplasts and tobacco ferredoxin under illumination.

Thus, as far as the reduction of PCA is concerned, a role of light in the biosynthesis of proline seems only to reduce NADP to NADPH with the non-cyclic electron flow system in photosynthesis.

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13) E. Adams and A. Goldstone, *J. Biol. Chem.*, **235**, 3499 (1960).