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Δ^1-Pyrroline-5-carboxylic Acid Dehydrogenase in Barley, a Proline-accumulating Species[1]

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ABSTRACT

The characteristics of the enzyme Δ^1-pyrroline-5-carboxylic acid dehydrogenase from etiolated barley (*Hordeum distichum*) shoots have been examined. The bulk of the enzyme activity was found in the 10,000*g* pellet fraction, this activity being displayed only after detergent treatment of the suspended pellet. The enzyme was most active at pH 8, and activity was NAD-dependent. Enzyme activity was unaffected by either mannitol or sucrose in the reaction mixture up to a concentration of 0.45 M but was strongly inhibited by Cl^- and, to a lesser extent, SO_4^{2-}. The inhibition attributable to KCl was reversed by increasing the concentration of Δ^1-pyrroline-5-carboxylic acid in the reaction mixture.

The enzyme Δ^1-pyrroline-5-carboxylic acid dehydrogenase catalyzes the second step of proline oxidation, the conversion of P5C[3] to glutamic acid. Crude preparations of the enzyme have been reported from mammalian liver (18, 19), bacteria (5, 6), and yeast (10, 11). Little is known of the enzyme from higher plants, the report of Stewart and Lai (15) being the first on this enzyme in such tissue. These authors, however, used tissues that do not accumulate proline when water stressed, and properties pertinent to stress conditions were not determined. The possibility that proline accumulation might arise from an inhibition of proline oxidation (1) led us to examine the enzyme from etiolated barley shoots, a tissue capable of proline accumulation (14).

MATERIALS AND METHODS

Growth of Plants. Barley (*Hordeum distichum* cv. Prior) seeds were soaked overnight on moist filter paper in Petri dishes and planted in pots of sand or vermiculite wet with half-strength Hoagland's solution. Seedlings grew in a dark chamber for 4 to 10 days at 25 C. Further watering was not required. Shoots of seedlings were excised near sand level, rinsed in distilled H_2O, blotted dry, and chilled before extraction.

Preparation of Extract. Etiolated shoots were cut into convenient lengths and ground with an ice-cold mortar and pestle in the cold room (2–4 C) with 10 volumes (w/v) of grinding medium (20) consisting of phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.3, 0.15 M) containing 0.25 M sucrose and 0.2% BSA (Sigma fraction V). Insoluble PVP (Polyclar AT) was also included in the grinding medium at 0.1 g/g of fresh plant material. The suspension was filtered and squeezed through two layers of cheesecloth, and centrifuged for 10 min at 1,000*g* (5 C). The supernatant was then centrifuged at 10,000*g* for 10 min (5 C), and the pellet, which contained most of the enzyme activity, was rinsed once with a small volume of grinding medium. The pellet was resuspended in 1 ml of grinding medium for each gram of fresh plant material and used without further purification. Little enzyme activity was lost when resuspended material was recentrifuged and suspended in grinding medium once more.

Preparation of Substrate. P5C was prepared by oxidation of proline by unwashed rat liver mitochondria, substantially as described by Johnson and Strecker (9). A typical incubation mixture contained 500 μmoles of L-proline, 1,000 μmoles of K-phosphate buffer, pH 7, and 40 to 50 mg of mitochondrial protein in a total volume of 25 ml. After 3 hr at 37 C, the yield of P5C was about 100 μmoles as estimated by the *o*-aminobenzaldehyde assay of Strecker (16). L-Proline-U-^{14}C (Amersham, 10 mCi/mmole) was used when ^{14}C-P5C was required. The reaction mixture was deproteinized by the addition of an equal volume of 10% trichloroacetic acid, and denatured protein was removed by centrifugation. Trichloroacetic acid was removed by repeated extraction with diethyl ether, until the pH had risen to 5. The P5C-containing solution was then adsorbed on a column of Dowex 50 (H^+). Once loaded, the column was washed with deionized water and then eluted in the cold room with 0.5 N HCl. Fractions were collected automatically. Figure 1 shows that this procedure gave good separation of P5C from ^{14}C-glutamic acid and ^{3}H-proline added as markers. In the experiment shown, proline was eluted with 2.5 N HCl; the increase in acid concentration was not necessary. That the OAB-positive material was P5C and not P2C (Δ^1-pyrroline-2-carboxylic acid) was indicated by its moving further than proline when chromatographed on paper or cellulose thin-layers in 1-butanol-acetic acid-water (4:1:5, v/v/v) and by its pink reaction with ninhydrin (0.25 g of ninhydrin, 2 ml of acetic acid, 98 ml of acetone). P2C runs slower than proline in this solvent and gives a purplish ninhydrin reaction (9, 12). Some preparations of P5C were purified further by paper chromatography in 1-butanol-acetic acid-water (4:1:5, v/v/v); this was not routinely done, however, as the material was equally effective as a substrate before and after the paper chromatographic purification. P5C was stored in 0.1 N HCl at 5 C and was stable at least two months under these conditions.

Enzyme Assay. P5C dehydrogenase was assayed by following the increase in absorbance at 340 nm. Cuvettes contained

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[3] Abbreviations: P5C: Δ^1-pyrroline-5-carboxylic acid; OAB: O-aminobenzaldehyde.

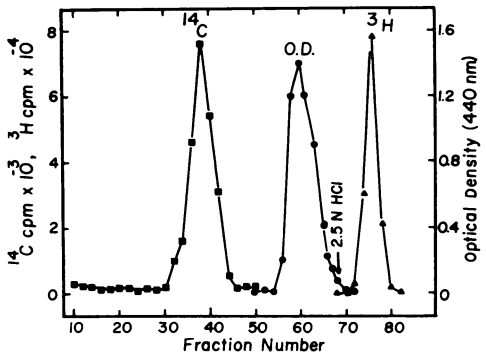

FIG. 1. Elution of P5C, ^{14}C-glutamate, and ^{3}H-proline from Dowex 50 (H^+) column. After deproteinization and removal of trichloroacetic acid, a P5C-containing reaction mixture (originally containing L-proline and rat liver mitochondria) was mixed with standard tritiated proline and ^{14}C-glutamic acid and loaded onto a column (20 cm × 0.9 cm) of Dowex 50 (H^+ form). Noncationic compounds were washed off the column with deionized water, and elution with 0.5 N HCl was begun. Aliquots of alternate fractions were counted by liquid scintillation or assayed for P5C by the OAB assay. Concentration of eluting acid was increased to 2.5 N after OAB-positive fractions had emerged.

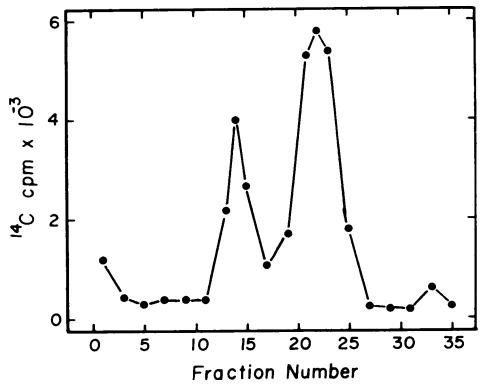

FIG. 2. Elution of deproteinized incubation mixture from Dowex 50 (H^+). Conditions were as in Figure 1 except that the reaction mixture contained ^{14}C-P5C, NAD, and P5C dehydrogenase preparation from etiolated barley.

100 μmoles of K-phosphate or Tricine buffer, pH 8; 1 μmole of P5C; 0.75 μmole of NAD; 0.8% Triton X-100, v/v; 0.1 ml of enzyme preparation, and water to give a final volume of 1.25 ml. Assays were conducted at 30 C.

Identity of Reaction Product. ^{14}C-P5C was included in the usual assay mixture and the reaction was stopped with trichloroacetic acid after NAD reduction had stabilized. After removal of protein and trichloroacetic acid, the mixture was chromatographed on Dowex 50 as described above. Figure 2 shows that two radioactive peaks emerged. The second peak was OAB-positive, indicating that it was unreacted substrate. The first peak contained ninhydrin-positive and radioactive material that was retained on Dowex 1 (acetate) and eluted with 0.5 N acetic acid, eliminating common amino compounds except aspartate, glutamate, tryptophan, and cysteic acid (8). Material from the Dowex 1 (acetate) column was chromatographed on cellulose thin layers in a two-dimensional solvent system which separates glutamic acid from all of these, and an autoradiograph was made of the developed plate. The first dimension was the isobutyric acid-based solvent of Crowley *et al.* (4), and the second solvent was 1-butanol-acetic acid-water (12:3:5, v/v/v). The major radioactive spot on the x-ray film (containing more than 90% of the total radioactivity) coincided perfectly with the ninhydrin-positive spot of standard glutamic acid. A smaller, unknown radioactive spot was also associated with a faint ninhydrin-positive spot. A trace of radioactivity was also associated with a ninhydrin-negative area of the chromatogram near glutamic acid. Although we have not identified these two minor radioactive products, it seems clear that the major product of the reaction is glutamic acid.

RESULTS

General Characteristics. Little activity could be demonstrated in crude homogenates, and the activity in the 10,000*g* pellet was completely dependent on treatment with Triton X-100 (0.5-1% v/v) or Tween 20 at the same concentration. Detergents were added directly to cuvette; the same volume of water did not increase enzyme activity. Activity of the detergent-treated enzyme was dependent on both NAD and P5C. The detergent treatment is apparently necessary to make the enzyme available to the substrate, rather than functioning in the catayltic mechanism, inasmuch as the fraction of activity that remains in the 100,000*g* supernatant was not increased by detergent. Moreover, when the isolation medium was buffered with HEPES (0.15 M) rather than phosphate, the detergent requirement was partially relieved.

Distribution of Enzyme Activity in Cell Fractions. In experiments with a number of preparations, it was found in all but one case that more than half (40–80%) of the total activity was contained in the 10,000*g* fraction. Most of the remaining activity (20–60%) was still in the supernatant even after centrifugation at 100,000*g* for 1 hr, indicating that the enzyme is not microsomal. Neither the 1,000*g* pellet, nor the 100,000*g* pellet contained more than 5% of the total activity in any preparation. Since it is to be expected that isolation of an organelle will result in a degree of loss of its enzymatic complement, the finding that 80% of P5C dehydrogenase activity was on some occasions associated with the 10,000*g* pellet probably means that all or almost all of the enzyme is particulate, and probably mitochondrial *in vivo*. This would be in agreement with the findings of other workers using plant (15), liver (19), and yeast (10) cells. This does not eliminate, of course, the possibility that *in vivo* the cell contains some enzyme in a nonparticulate fraction.

Response to pH. The enzyme was most active at about pH 8 in both phosphate and Tricine buffers (Fig. 3). Similar re-

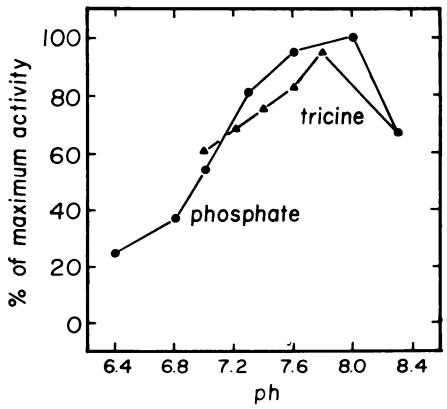

FIG. 3. Response of barley P5C dehydrogenase activity to pH in two buffers. Assays were conducted under standard conditions except that buffers were adjusted previously to pH values shown. In this experiment, relative velocity of 100 = 3.2 nmole of NADH formed/min·mg of protein.

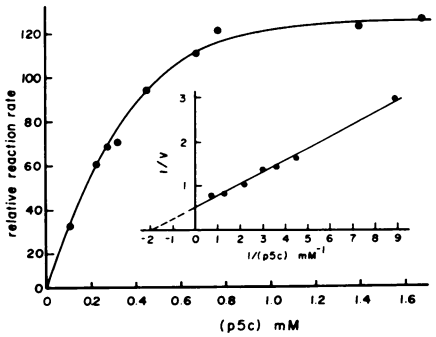

FIG. 4. Response of barley P5C dehydrogenase activity to P5C concentration. Assays were conducted under standard conditions except for variations in P5C concentration. In this experiment, relative velocity of 100 = 4.2 nmoles of NADH formed/min·mg of protein.

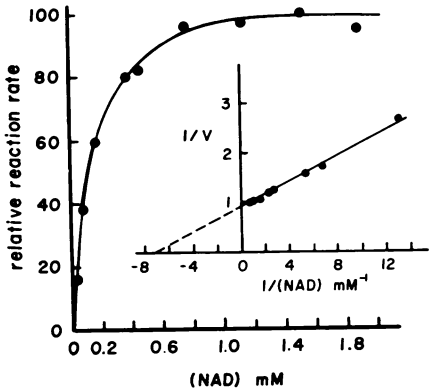

FIG. 5. Response of barley P5C dehydrogenase activity to NAD concentration. Assays were conducted under standard conditions except for variations in NAD concentration. In this experiment, relative velocity of 100 = 4.0 nmoles of NADH formed/min·mg of protein.

sponses were shown in tris and glycyl-glycine buffers, but total activity was considerably reduced in these buffers.

Response to Substrate Concentration. At a saturating concentration of NAD, the response of enyzme activity to P5C concentration was hyperbolic (Fig. 4), and the enzyme had a high affinity for the substrate. We have calculated apparent *Km* values of 0.2 to 0.5 mM from this type of data. The two plots in Figure 4 are from different experiments, and somewhat different *Km* values can be obtained from them. The absolute value is in any case subject to error, however, as one cannot be sure that the amount of P5C measured by the OAB assay is all available to the enzyme. Strecker (17) found that his purest preparations of P5C were enzymatically converted to proline in only 80% yield, and that stability of the compound as measured by the OAB assay was greater than when measured by enzymatic activity. The source of this difficulty may be that P5C forms polymeric products that are not available to enzymes, while still being OAB-positive. Figure 5 shows similar data obtained by varying the NAD concentration. The calculated *Km* in this case is 0.15 M. Although the data shown here were obtained at a nonsaturating concentration of P5C, a similar value for *Km* was obtained when the experiment was done at a saturating P5C concentration.

Effect of Various Solutes. As barley accumulates proline under conditions of water deficit (14) and salinity (2), it was of interest to test the effect of water potential and salts on the enzyme. Nonionic solutes such as sucrose or mannitol had little influence on activity (Fig. 6). Activity was markedly reduced by rather low concentrations of several salts, particularly by chlorides. Seventy-five per cent inhibition by potassium, sodium, and ammonium chlorides occurred at concentrations of 0.2 to 0.25 M or less, and magnesium chloride was even more drastically inhibitory. Concentrations of the last salt higher than 0.04 M resulted in a precipitate (presumedly magnesium phosphate) that invalidated the optical assay method, even when the reaction mixture was buffered with Tricine. The corresponding sulfates, while still inhibitory, were markedly less so, indicating that the effect was not simply one of ionic strength, but that chloride ion is of particular significance. The finding of Greenway and Sims (7), that salt inhibition of malate dehydrognase can be reversed by high substrate concentration, led us to check the effect of increased P5C concentration in the presence of KCl. Table I shows that the inhibitory effect of KCl is relieved by increasing P5C concentration above normally saturating levels. In this experiment, maximum velocity was 3.8 nmoles of NADH formed/min·mg of protein.

DISCUSSION

The characteristics of the enzyme in barley shoot tissue, a potential proline-accumulating system, are essentially similar to those of the enzyme described from nonaccumulating plant tissues and other organisms (5, 6, 10, 11, 15, 18). The activity of this enzyme is a potential source of control for endogenous free proline concentration in the tissue, and inhibition of its activity could account, at least partially, for the rapid accumulation of proline recorded in many species during water, salt,

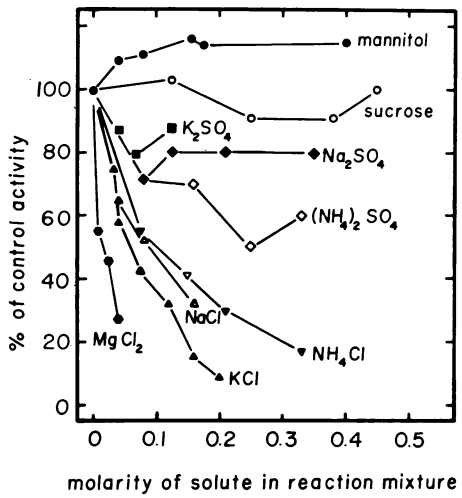

FIG. 6. Response of barley P5C dehydrogenase activity to various solutes. Assays were conducted under standard conditions except for inclusion of solutes shown. When $MgCl_2$ was used, reaction mixture was buffered with Tricine rather than phosphate.

Table I. *Removal of Inhibition Attributable to KCl by Increased P5C Concentrations*

KCl Treatment	P5C Concn	Relative Activity
M	*mM*	
Control	0.56	100
0.16	0.56	43
0.16	1.08	77
0.16	2.16	108
0.33	0.56	18
0.33	1.08	43
0.33	3.24	71

or cold stress (2, 3, 14). In this connection, the inhibition of enzyme activity in the presence of various ions appears significant. It is apparent that osmotic potential *per se* was without effect on the activity of the enzyme and that the inhibition was attributable to the presence of the ions, particularly Cl^-. Although this response could account for proline accumulation in salt-stressed plants, the case for control in water- or cold-stressed plants is more tenuous. Proline accumulation in dehydrated tissues commences when 30% or less of total tissue water has been lost (14), a loss that is unlikely to concentrate salt solutions within the cells to a level inhibitory to P5C dehydrogenase activity. Proline accumulates less rapidly in plants exposed to NaCl in the rooting medium than in those exposed to polyethylene glycol solutions of similar osmotic potential (2). As the internal chloride status of the former plants will undoubtedly be higher than the latter, initiation of proline accumulation following chloride inhibition of P5C dehydrogenase seems an unlikely explanation for the polyethylene glycol-stimulated accumulation of proline.

The data provided in this report support the conclusion from other studies (15) that this enzyme is located in the mitochondrion. Moreover, the enzyme seemed to be unavailable to supplied substrate unless treated with detergent. Although this may be an artifact of the cell-free system, it raises the possibility that access of the enzyme to its substrate is a potential point of control of the rate of the reaction. Further work on the other enzymes in the biosynthetic (13) and oxidative (12, 15) pathways of proline is required before it can be usefully predicted whether permeability may be a factor in the control of proline accumulation.

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