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L-PROLINE DEHYDROGENASE OF *TRITICUM VULGARE* GERM: PURIFICATION, PROPERTIES AND COFACTOR INTERACTIONS*

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Abstract—The enzyme catalysing the L-proline-dependent reduction of NAD^+ has been purified over 600-fold from wheat germ acetone powder extracts. L-Proline, 3,4 dehydro-DL-proline, thiazolidine-4-carboxylate were the only substrates utilized readily. The K_m for L-proline was 1·0 mM and for NAD^+ 0·8 mM. The enzyme was highly specific for NAD^+ with $NADP^+$ and NADPH acting as effective competitive inhibitors with a K_i of 1·8 and 0·4 μM, respectively. All ribonucleoside triphosphates tested were good non-competitive inhibitors, in particular UTP. The purified enzyme could reduce pyrroline-5-carboxylate, either chemically synthesized or generated in a linked assay system from ornithine by a highly-purified ornithine transaminase. In the latter case both NADH and NADPH were utilized equally well as the reductant. With chemically synthesized DL-pyrroline-5-carboxylate as the substrate, NADPH was used at only 25% the rate of NADH, and NADPH strongly inhibited the oxidation of NADH.

INTRODUCTION

THE BIOSYNTHESIS of proline in all organisms so far studied in any detail proceeds from either glutamic acid or ornithine via glutamic-γ-semialdehyde which is in equilibrium with its cyclic form P5C.† This latter compound is then reduced to L-proline, utilizing a reduced pyridine nucleotide as the reductant.[1] The oxidation of L-proline on the other hand has been accomplished by the L-amino acid oxidase of animal tissues,[2] and the particulate proline oxidases of rat liver,[3,4] yeast,[5] and insect flight muscle.[6] No requirement for pyridine nucleotide has been demonstrated for the oxidation of proline in these cases, and the P5C reductase of calf liver was incapable of the reverse reaction.[7] Recently, a dehydrogenase has been obtained from extracts of acetone powders of germinating peanut cotyledons which can reduce NAD^+ in the presence of L-proline.[8] The specificity for NAD^+ was very great. $NADP^+$ could not replace NAD^+ but was an extremely effective competitive inhibitor of the NAD^+-linked reaction. The same extracts also contained a P5C reductase which utilized NADPH much more effectively than NADH.[9] The dehydrogenase activity did not appear to be, therefore, merely a reversal of the reductase.

* Part III in the series "Metabolism of Proline in Higher Plants". For Part II see Ref. 8.

† *Abbreviations*: P5C-Δ-pyrroline-5-carboxylate; CAPS-cyclohexylamino propane sulfonic acid.

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[8] MAZELIS, M. and FOWDEN, L. (1971) *J. Exp. Botany* **22**, 137.

[9] MAZELIS, M. and FOWDEN, L. (1969) *Phytochemistry* **8**, 801.

We have recently found that wheat germ also contains an NAD^+-linked L-proline dehydrogenase.[10] The present report describes the isolation, partial purification and properties of this enzyme. There is a specific requirement for NAD^+ as the oxidant. $NADP^+$ is a very powerful competitive inhibitor. Even more effective, however, is NADPH. An unusual finding has been the ability of the enzyme to reduce P5C to proline with either NADH or NADPH as the reductant. Thus the paradoxical situation apparently exists of a reversible reaction in which the cofactor which is operative in one direction is not only inoperative, but inhibitory in the reverse direction. Some possible explanations of this phenomenon are offered in the discussion.

RESULTS

Purification of the enzyme

The enzyme was extracted from the acetone powder with 5 vol. (w/v) of 0·2 M potassium phosphate buffer pH 7·2 by stirring in the cold for 30 min. The suspension was passed through several layers of cheesecloth and centrifuged for 60 min at 23 000 *g*. The supernatant solution was used as the starting point for purification. All subsequent steps were carried out in the cold except where noted.

The enzyme solution was made to 50% saturation with solid $(NH_4)_2SO_4$ and stirred for 30 min. The precipitate was removed by centrifugation and dissolved in 0·1 M phosphate pH 7·2. All of the enzyme activity was recovered in this fraction. The enzyme solution was then heated at 60° for 3 min and the precipitate which formed was removed by centrifugation and discarded.

The heat-treated enzyme solution was placed on a column of Sephadex G100 and eluted from the column with 0·01 M phosphate pH 7·2 as the eluting solvent. Approximately 10 ml aliquots were collected. The enzyme appeared immediately at the void volume. All aliquots having specific activities at least twice that of the material applied to the column were combined. The combined solution was concentrated by ultra-filtration using a filter which retained proteins with a MW greater than 30 000. An inexplicable observation was the fact that when Sephadex G200 was used in this step, very little activity could be recovered in any of the fractions. Recombination of fractions did not give additional activity. Any activity that was recovered, however, also appeared immediately after the emergence of the void volume with this gel.

The concentrated eluate from the preceding step was placed on a hydroxylapatite column (22 × 1·2 cm). The column was eluted with 0·02 M phosphate pH 7·2 collecting 5 ml fractions until a base level of absorbance at 280 nm was reached. At this point a gradient elution was started with 0·5 M phosphate pH 7·2 and a mixing vessel containing 150 ml of 0·02 M phosphate pH 7·2 originally. The fractions containing the activity were combined and dialysed against 0·02 M K phosphate buffer, pH 7·2, and then concentrated to 10 ml by ultra-filtration.

The concentrated enzyme solution was rechromatographed on an identical hydroxylapatite column and eluted using the same conditions as in the preceding step. The fractions containing the enzyme (between 200 and 220 ml as a sharp peak) were kept individually for further use. The results of a typical purification are given in Table 1. The activity given for the second hydroxylapatite elution is based on the addition of all the separate fractions

[10] MAZELIS, M. and CREVELING, R. K. (1972) *Plant Physiol.* **49** suppl. 26.

and their average specific activity. The fraction with the highest activity was purified 640-fold.

TABLE 1. PURIFICATION OF L-PROLINE DEHYDROGENASE

Fraction	mg protein	Sp. act. (units/mg)	Total units	Purification	Yield
1 Acetone Powder extract	3568	0·013	46·6	1·0	100
2 0–50% $(NH_4)_2SO_4$ precipitate	2409	0·018	43·4	1·4	93
3 Heated $(NH_4)_2SO_4$ fraction	675	0·059	39·8	4·5	86
4 Sephadex G100 conc. eluate	252	0·20	50·4	15	108
5 First hydroxylapatite eluate	14	2·2	30·8	169	66
6 Second hydroxylapatite eluate	3·4	5·6	19·1	430	41

Kinetic constants

The Michaelis constants for NAD^+ and L-proline were determined using the most purified fraction. The K_m for each substrate was obtained in the presence of saturating concentrations of the other substrate. The reactions followed a normal hyperbolic curve in each case. By graphical methods the K_m obtained for L-proline was 1·0 mM and for NAD^+ was 0·8 mM.

Substrate specificity for proline

The specificity of the reaction for the amino acid was examined (Table 2). The enzyme was completely specific for L-proline with D-proline being neither a substrate nor an inhibitor. Hydroxyproline was not a substrate. 3,4-Dehydro-DL-proline was even more effective than L-proline. Thiazolidine-4-carboxylate, a sulfur-containing analog of proline, was utilized as effectively as proline. *N*-Substitution of the proline eliminated its ability to act as a substrate, and modifying the carboxyl group of proline decreased or eliminated the substrate function completely.

TABLE 2. SUBSTRATE SPECIFICITY OF L-PROLINE DEHYDROGENASE

Substrate	Relative activity	Substrate	Relative activity
L-Proline	100	L-Proline methyl ester	48
D-Proline	0	N-Methyl-L-proline	0
DL-Proline	109	L-Proline amide	0
4-Hydroxy-L-proline	0	DL-Pipecolic acid	19
3-*trans*-Hydroxy-L-proline	2	Thiazolidine-4-Carboxylate	111
3,4-Dehydro-DL-proline	150		

The standard reaction mixture was utilized with the substrate designated. In the cases where DL-substrates were used, the concentration was raised so the L-form was at the equivalent concentration to that used in the L-proline assay.

Pyridine nucleotide specificity

The reaction with proline was found to be completely specific for NAD^+ as the electron acceptor. $NADP^+$ could not replace NAD^+ to any degree. In fact, $NADP^+$ was found to be an extremely effective competitive inhibitor for NAD^+. $NADPH^+$ was also a competitive inhibitor for NAD^+ and was even more potent than $NADP^+$. The K_i for $NADP^+$ and also for $NADPH^+$ was determined by graphical methods. The K_i for $NADP^+$ was 1·8 μM and for $NADPH^+$ was 0·4 μM. The addition of $NADPH^+$ to a final concentration of 5 μM in a reaction mixture of 9 mM NAD^+ inhibited the rate by 80%. It required 20 μM $NADP^+$ to achieve the same degree of inhibition. NADH did not show any inhibitory effects on the initial rate of reaction with NAD^+.

Inhibitory effects of nucleotides

Other nucleotides were tested for their effect on the oxidation of proline by NAD^+. The results (Table 3) clearly show that all the ribonucleotide triphosphates tested were good inhibitors. At 2 mM final concentration an inhibition of between 70 and 80% was found for all except UTP. This latter nucleotide gave almost complete inhibition at this concentration, and 77% inhibition was found at a concentration as low as 0·5 mM. At the 2 mM concentration ADP was less effective inhibiting only 40% and AMP only gave a 20% inhibition. ATP and UTP were examined in more detail to determine the mode of inhibition. The results indicate that both are non-competitive inhibitors with respect to NAD^+.

TABLE 3. NUCLEOTIDE TRIPHOSPHATE INHIBITION OF L-PROLINE DEHYDROGENASE

Nucleotide	Relative activity	Nucleotide	Relative activity
None	100	GTP	31
ATP	31	CTP	22
ITP	21	UTP	2

The standard reaction mixture was used as described in Experimental with the addition of the designated nucleotide to a final concentration of 2 mM.

Product of the reaction and reversibility

Because of the rapid decrease in the rate of reaction with time, the product of the oxidation from L-proline did not accumulate in any quantity under the reaction conditions used. Even with ^{14}C-proline as the substrate too little was found for identification.

The biosynthesis of proline involves the reduction of P5C by reduced pyridine nucleotide.[1] Synthetic P5C was obtained from DL-α-amino-δ-hydroxy valeric acid as described in the Experimental and tested with the L-proline dehydrogenase. Both NADH and NADPH were oxidized in this system. NADPH was less effective being oxidized at only 25% the rate of NADH. Both $NADP^+$ and NADPH were excellent inhibitors of the NADH reduction of P5C. $NADP^+$ at 10 μM inhibited the reaction by 64% and 1 μM NADPH inhibited the rate by 56%.

Another method of P5C synthesis was by the enzymatic transamination of L-ornithine. An ornithine-δ-transaminase purified some 430-fold from squash cotyledons of 5–8-day-old etiolated seedlings was available.[11,12] This enzyme produces glutamic γ-semialdehyde

[11] MAZELIS, M. and LU, T. S. (1971) *Plant Physiol.* **47**, suppl. 17.
[12] MAZELIS, M. and LU, T. S. (1973) *Federation Proc.* **32**, 589.

and its equilibrium product P5C by transamination with α-oxoglutarate. A linked reaction system combining the ornithine transaminase of squash cotyledons and the wheat germ dehydrogenase was set up as described in the Experimental. The system oxidized both NADH and NADPH equally effectively as shown in Fig. 1. In the absence of either the dehydrogenase or the transaminase no oxidation occurred. DL-Ornithine-2-^{14}C was used as the substrate with NADH or NADPH as the reductant and the reaction mixture examined by paper chromatography. The radioactive compounds were located by use of a strip counter and identified by co-chromatography with known standards. With either NADH or NADPH as the reductant, radioactive proline was formed. In the absence of the transaminase or the dehydrogenase essentially no proline was found.

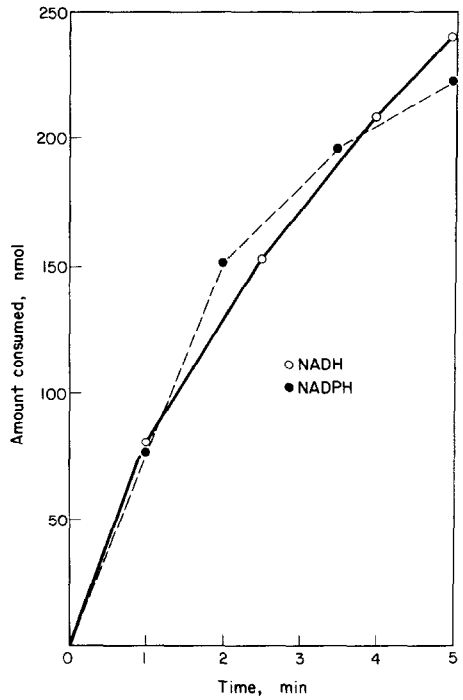

FIG. 1. OXIDATION OF REDUCED PYRIDINE NUCLEOTIDES BY ENZYMATICALLY-PRODUCED P5C. Assay mixture as described in Experimental except pyridine nucleotide concentration was 0·25 mM.

DISCUSSION

Previous studies had shown that a system from germinating peanut cotyledons could synthesize proline via transamination of ornithine and reduction of the product by reduced pyridine nucleotides.[9] The same preparation could also reduce pyridine nucleotides in the presence of L-proline.[8] The biosynthesis of proline utilized NADPH more effectively than NADH. A partially purified P5C reductase from tobacco leaves also utilized NADPH more readily.[13] In contrast to this a proline dehydrogenase from peanut cotyledons used NAD^+ as an oxidant for L-proline to a much greater extent than $NADP^+$.[8] In fact $NADP^+$ was an excellent competitive inhibitor of NAD^+.

[13] NOGUCHI, M., KOIWAI, A. and TAMAKI, E. (1966) *Agr. Biol. Chem.* **30**, 452.

The present work shows that many of the findings of the properties of the L-proline dehydrogenase of peanut cotyledons are equally applicable to a similar enzyme from wheat germ. The K_m for L-proline and NAD^+ of this latter enzyme are more than an order of magnitude larger than that of the peanut enzyme. NAD^+ is almost completely specific as the oxidant, and $NADP^+$ is an excellent competitive inhibitor. In addition NADPH is even more effective as an inhibitor of the wheat germ enzyme than $NADP^+$. Nucleotide triphosphates, especially UTP, have been found to be good non-competitive inhibitors of the dehydrogenase. Strangely enough P5C, which is formed by transamination from ornithine, is reduced equally well to proline with either NADPH or NADH; however, chemically synthesized P5C uses NADH much more effectively than NADPH. A similar finding was reported for the P5C reductase of pumpkin (*Cucurbita maxima*) cotyledons.[14] The oxidation of NADH by the wheat germ dehydrogenase using DL-P5C produced through chemical synthesis was inhibited very markedly by the presence of small amounts of NADP or NADPH.

How can we put these seemingly contradictory observations together to make a logical whole? Attempts to determine the MW by gel-filtration have indicated that the MW of the dehydrogenase is of the order of 200 000 daltons. Proteins of this size are almost invariably composed of sub-units. Let us assume that there is a binding site for P5C of high affinity used in biosynthesis, and another of high affinity for proline used in the oxidation of proline. It is assumed that the high affinity site for P5C would have a low affinity for proline. Again assume there is only one binding site for the pyridine nucleotides and that the binding constants for the pyridine nucleotides are of the order $NADPH > NADP^+ > NADH$ and NAD^+ and that the presence of NADPH or $NADP^+$ on the pyridine nucleotide site makes the high affinity proline site much less able to bind P5C. We can now rationalize all the preceding results into a consistent picture. With NADH as the reductant and starting with high amounts of chemically-synthesized P5C the substrate can bind to both sites and is reduced to proline. In the presence of NADPH and NADP the second site becomes less available to P5C and the rate with NADH is decreased. The high affinity P5C site can still be used so NADPH can reduce P5C at this site. When P5C is furnished via the transaminase and is reduced as it is formed, it binds primarily to the high affinity site and the rate of reduction with NADPH and NADH are the same. Implicit in the above proposed mechanism is the assumption that NADPH and $NADP^+$ only can interact with the high affinity site for P5C and $NADH^+$ and NAD^+ can react with both sites.

Evidence has recently been accumulating that there are cases where two binding sites for substrate on the same protein does exist. Smith *et al.*[15] have shown separate sites for oxidation and reduction on cytochrome C and its interaction with cytochrome oxidase and succinate-cytochrome C reductase. Bosshard[16] has also presented evidence that subtilisin may have two different substrate binding sites.

EXPERIMENTAL

Enzyme assays.—For proline oxidation: The reaction was carried out in silica cuvettes containing the following components in a final vol. of 1 ml: CAPS, pH 10·3, 70 mM; L-proline 10 mM; NAD^+, 9 mM; enzyme. The reaction was started by the addition of enzyme and the rate determined by the increase in absorbance at 340 nm using a recording spectrophotometer.

[14] SPLITTSTOESSER, S. A. and SPLITTSTOESSER, W. E. (1973) *Phytochemistry* **12**, 1565.
[15] SMITH, L., DAVIES, H. C., REICHLIN, M. and MARGOLIASH, E. (1973) *J. Biol. Chem.* **248**, 237.
[16] BOSSHARD, H. R. (1973) *FEBS Letters* **30**, 105.

For P5C reduction. Two methods were used. (a) A direct determination of the oxidation of reduced pyridine nucleotide was made using the following reaction mixture: K phosphate, pH 7·2, 100 mM; NADH or NADPH, 0·4 mM; P5C, 10 mM; wheat germ dehydrogenase, 0·02–0·05 international units (IU) sp. act. 3·5 IU/mg. Final vol. was 1 ml. The oxidation of the reduced pyridine nucleotide was followed at 340 nm. (b) Linked assay system: Tris/HCl pH 8·0, 150 mM; Na α-oxoglutarate, 50 mM; L-ornithine, 50 mM; pyridoxal-5'-phosphate, 0·05 mM; NADH or NADPH, 0·4 mM; orinithine transaminase, 0·04–0·08 IU (sp. act. 2·6 IU/mg); Wheat germ dehydrogenase, 0·02–0·05 IU (sp. act. 3·5 IU/mg); Final vol. 1 ml. The ornithine transaminase was prepared from squash (*Cucurbita pepo*) cotyledons and was over 400-fold purified.[12] The reaction was started by the addition of the transaminase to the reaction mixture and the progress followed by the change in absorbance at 340 nm.

Product of P5C reduction. To identify the product of the above linked enzyme system, the following reaction mixture was used: Tris/HCl, 50 mM; L-ornithine, 20 mM; DL-ornithine-2-^{14}C, 20 mM containing 10 μCi^{14}C; Na α-oxoglutarate, 40 mM; NADH, or NADPH, wheat germ dehydrogenase and ornithine transaminase at same levels as above. Final vol. 0·5 ml. After 60 min of incubation at room temp. the reaction was terminated with 1 ml 95% EtOH. The mixture was clarified by centrifugation and the supernatant examined by PC followed by strip counting and co-chromatography with known standards.

Protein determination. Protein was estimated by use of either the Lowry colorimetric or UV-spectrophotometric methods as described by Layne.[17]

Preparation of acetone powder. Wheat (*Triticum vulgare*) germ was a gift of Prof. P. K. Stumpf. The wheat germ was blended for 1 min with 4–5 vols. (w/v) of acetone (at −15°) in a chilled Waring blendor. The suspension was filtered by suction and washed well with acetone. The precipitate was then dried at room temp. and stored at −15°. The powder was stable for many months.

Synthesis of P5C. P5C was prepared from DL-α amino-δ-hydroxyvaleric acid by a slight modification[18] of the method used by Jones and Broquist.[19] The concentration of the product was estimated by the assay method of Strecker.[20]

Chemicals. 3,4-Dehydro-DL-proline and 3-*trans*-hydroxy-L-proline were gifts from Prof. L. Fowden. All other amino acids and other chemicals were obtained commercially.

Acknowledgement—This work was partially supported by Grant AP00699 from the Air Pollution Control Office and NASA Contract NAS2-6633.

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